Physiological responses of Nile tilapia (*Oreochromis niloticus*) after exposure to diclofenac and metoprolol

DISSERTATION

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Erklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit selbständig und nur unter Verwendung der angegebenen Literatur und Hilfsmittel angefertigt habe. Des Weiteren erkläre ich meine Kenntnisnahme der dem angestrebten Verfahren zugrunde liegenden Promotionsordnung. Ich habe mich anderweitig nicht um einen Doktorgrad beworben und bin nicht im Besitz eines entsprechenden Doktorgrades.

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List of abbreviations and acronyms

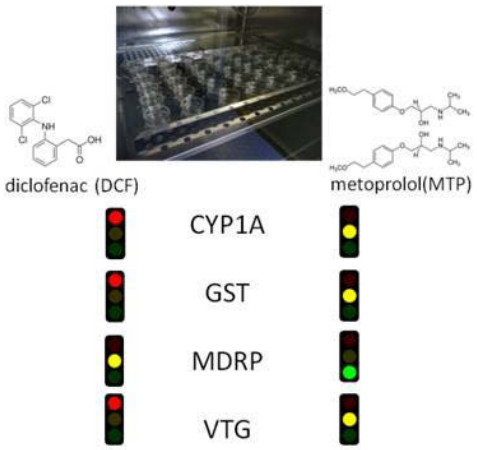
ABC protein	ATP-binding cassette protein
AFW	artificial fresh water
AMV-RT	avian myeloblastosis virus reverse transcriptase
ANOVA	analysis of variance
aqua dest.	aqua destillata
ATP	adenosine triphosphate
AR(s)	adrenoreceptor(s)
BLAST	basic local alignment search tool
bp	base pairs
°C	degree Celsius
Ca^{2+}	calcium
cAMP	cyclic adenosine monophosphate
CAS	chemical abstracts service
CAT	catalase
cDNA	complementary DNA
CI	condition index (according to Futon)
CMC	calcium-magnesium containing medium
CMF	calcium-magnesium free medium
COX	cyclooxygenase
C_T	cycle threshold
CYP	cytochrome P450
CYP1A	cytochrome P4501A monooxygenase (family 1, subfamily A)
DCF	diclofenac
DMSO	dimethylsulfoxide
DNA	desoxyribonucleic acid
DNase	desoxyribonuclease
dNTP	desoxynucleotide phosphate
dph	days post-hatch
DTT	dithiotreitol
EDC(s)	endocrine disrupting chemical(s)
EDTA	ethylenediaminetetra-acetate
E2	17β-estradiol
EE2	17α-ethinylestradiol

EF1-a	elongation factor 1-α
ER	estrogen receptor
EROD	ethoxyresorufin-O-deethylase
EtOH	ethanol
FAO	food and agriculture organization of the United Nations
FSH	follicle stimulating hormone
FTS	flow-through system
g	gram
g	gravitational acceleration
gDNA	genomic DNA
GH	growth hormone
GHRH	growth hormone releasing hormone
GnRH	gonadotropin-releasing hormone
GPx	glutathione peroxidase
GST	glutathione-S-transferase
h	hour
HCl	hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPG	hypothalamus-pituitary gonad
HSI	hepatosomatic index
hsp70	heat shock protein 70
IGF-I	insulin-like growth factor I
in. prep.	in preparation
11-KT	11-ketotestosterone
L	liter
LaGeSo	Landesamt für Gesundheit und Soziales, Berlin
LH	luteinizing hormone
LOEC	lowest observed effect concentration
LPO	lipid peroxidation
m	meter
M	molar
MDA	malondialdehyde
MDRP	multidrug resistance protein
MEM	minimum essential medium
mg	milligram
$MgCl_2$	magnesium chloride

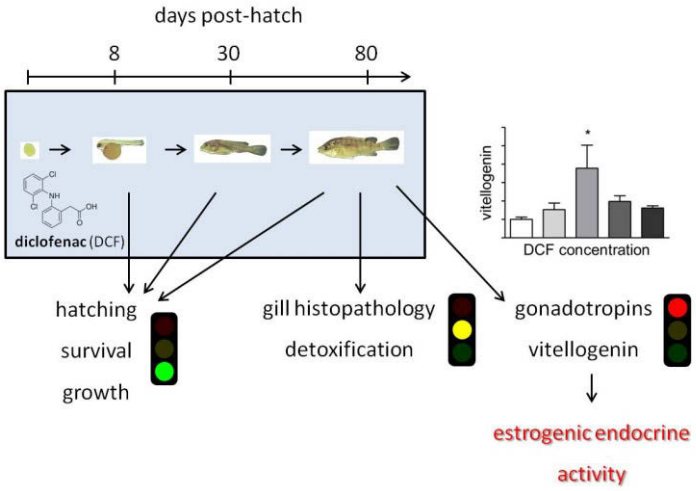
min	minute
mL	milliliter
µL	microliter
mm	millimeter
mM	millimolar
MMLV-RT	moloney murine leukemia virus - reverse transcriptase
mRNA	messenger ribonucleic acid
MS222	tricaine methanesulfonate
MTP	metoprolol
NaEDTA	tetrasodium ethylenediaminetetraacetate
NaP	sodium phosphate
NCBI	National Center of Biotechnology Information
ng	nanogram
NH_4^+	ammonium
NMDR	non monotonic dose response
nmol	nanomol
NO_2^-	nitrogen dioxide
NO_3^-	nitrate
NOEC	no observed effect concentration
NSAID(s)	non-steroidal anti-inflammatory drug(s)
NTC	no template control
OECD	Organisation for Economic Co-operation and Development
p.a.	pro analysis
PCR	polymerase chain reaction
per. comm.	personal communication
PGE_2	prostaglandin E_2
P-gp	permeability glycoprotein
PhAC(s)	pharmaceutical active compound(s)
PO_4^{3-}	phosphate
poly(dt)	poly desoxythymidine
PTFE	polytetrafluorethylen
qPCR	quantitative real time PCR
Q-TOF LC/MS	quadrupole time-of-flight liquid chromatography/mass spectrometry
RIN	RNA integrity number
RNA	ribonucleic acid
RNase	ribonuclease

ROS	reactive oxygen species
rpm	rotations/rounds per minute
rRNA	ribosomal RNA
RT	reverse transcriptase
s	second
SBP(s)	sex hormone binding protein(s)
SD	standard deviation
SDS	sodium dodecyl sulfate
SLs	secondary lamellae
SST	somatostatin
STP(s)	sewage treatment plant(s)
Ta	annealing temperature
TBA	thiobarbituric acid
TBARS	thiobarbituric acid substances
TCA	trichloroacetic acid
Tris	tris(hydroxymethyl)aminomethane
U	unit
UV	ultraviolet
VTG	vitellogenin
WHO	world health organization
w/v	[weight of solute (g) / volume of solution (mL)]

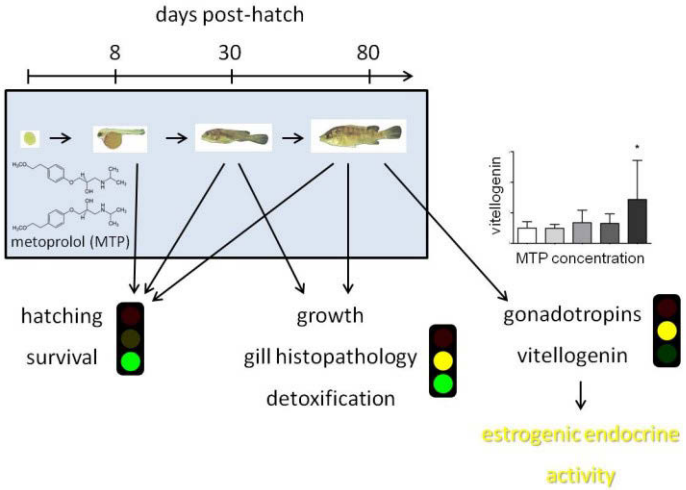
***In vitro* exposure**



***In vivo* DCF exposure**



***In vivo* MTP exposure**



Zusammenfassung

(Oberflächen-) Gewässer weltweit sind mit geringen Mengen (ng/L bis wenige µg/L) humaner Pharmazeutika belastet. Diclofenac (DCF; nicht-steroidal, entzündungshemmend) und Metoprolol (MTP; ß-Blocker) gehören entsprechend ihres hohen Verbrauchs zu den am häufigsten gefundenen Substanzen. Deren biologische Aktivität ist nicht auf den Menschen beschränkt. Gut konservierte Enzyme innerhalb der Vertebraten legen Auswirkungen auf Nicht-Zielorganismen wie Fische nahe, die bisher in Langzeituntersuchungen mit umweltrelevanten Konzentrationen unzureichend untersucht wurden. In der vorliegenden Arbeit wurden die physiologischen Effekte von DCF und MTP auf die Nil-Tilapie (Oreochromis niloticus), einem der wichtigsten Aquakulturfische weltweit, untersucht. In vitro konnte anhand primärer Hepatozyten gezeigt werden, dass bereits umweltrelevante Konzentrationen von DCF zu einer erhöhten Genexpression verschiedener Schlüsselenzyme der Detoxifizierung führten. Nach MTP-Exposition waren die Veränderungen weniger eindeutig. Beide Substanzen induzierten die Vitellogenin Genexpression, nur DCF jedoch bereits in umweltrelevanter Konzentration. In vivo wurden in zwei Langzeit-Expositionsversuchen die physiologischen Effekte vom befruchteten Ei bis 80 Tage nach Schlupf in O. niloticus untersucht. Beide Substanzen hatte keinen Einfluss auf Schlupferfolg und Überleben, das Wachstum war nach 80 Tagen nach Schlupf leicht reduziert. Die deutlichsten Auswirkungen waren histopathologische Veränderungen der Kiemen, veränderte Genexpressionen der Gonadotropine und eine erhöhte Expression von Vitellogenin. Die Ergebnisse legen eine stärkere östrogene Aktivität von DCF im Vergleich zu MTP nahe. Zusammenfassend sind die Bedenken gegenüber den Einzelsubstanzen eher gering, negative Auswirkungen auf die Reproduktion und sich verstärkende Effekte bei zeitgleicher Exposition gegenüber DCF und MTP lassen sich jedoch nicht ausschließen und sollten im Weiteren untersucht werden.

Schlagwörter: Pharmazeutika, Diclofenac, Metoprolol, Nil-Tilapie, primäre Hepatozyten, Langzeitexposition, populationsrelevante Endpunkte, Kiemen Histopathologie, Detoxifizierung, Gonadotropine, östrogene Aktivität

Summary

Surface waters worldwide are contaminated with low levels (ng/L up to few µg/L) of human pharmaceuticals. Diclofenac (DCF; non-steroidal, anti-inflammatory) and metoprolol (MTP; ß-blocker) are highly consumed and therefore commonly detected. Their biological activity is not restricted to humans. Well conserved enzymes within the vertebrates suggest effects on non-target organisms such as fish, poorly studied in long-term exposure experiments using environmentally relevant concentrations. In the presented work, physiological effects of DCF and MTP on the Nile tilapia (Oreochromis niloticus), an important aquaculture fish species, were studied. Using primary hepatocytes, it was shown in vitro that environmentally relevant concentrations of DCF increased the gene expression of different key enzymes of the detoxification, while MTP exposure had a less clear effect. Both substances induced vitellogenin gene expression, but only after DCF exposure this was significantly elevated already at the environmentally relevant concentration. In vivo, two long-term exposure studies on the physiological effects from the fertilized egg until 80 days post-hatch were evaluated. Both substances did not affect hatching success and survival, while growth was slightly reduced after 80 days post-hatch. Histopathological alterations of the gills, changed gene expression patterns of the gonadotropins and induced vitellogenin gene expression were the most dominant findings. The results indicate a stronger estrogenic mode of action of DCF compared to MTP. Overall, the risk due to a single substance exposure seems to be relatively low but adverse effects on reproduction and additive effects during simultaneous exposure to DCF and MTP cannot be excluded and should be investigated further.

Keywords: pharmaceuticals, diclofenac, metoprolol, Nile tilapia, primary hepatocytes, long-term exposure, general parameters, gill histopathology, detoxification, gonadotropins, estrogenic activity

1 Introduction

At present, hardly any freshwater ecosystem exists which is not directly or indirectly affected by human activities. With the progress of industrialization and the steady increase in the world´s population, human-induced stressors to aquatic ecosystems and the organisms living therein, increased. Encroachments range from structural changes of surface waters such as river straightening for vessel traffic to changes in the chemical composition of the water bodies. These can be due to accidental or intentional releases of liquid and solid waste products. Aquatic systems functioned as sinks for anthropogenic pollutants ever since and hence show a huge diversity of contaminants (Fent 2003). Besides growing industry, the increasing input of nutrients and chemicals can also be attributed to the growing agricultural sector, including aquaculture. Therefore, human pharmaceuticals, veterinary medical compounds and a large number of pesticides is frequently found in surface waters but also in ground water and occasionally even in drinking water (Heberer 2002; Fent et al. 2006). Aquatic organisms are likely impaired, since they are exposed throughout their life cycles, and as a feedback mechanism, humans might be as well when consuming aquatic organisms (Fent 2003).

1.1 Pharmaceuticals and their endocrine disruption potential

The occurrence of human pharmaceuticals and their metabolites in aquatic ecosystems is of growing international concern. A great variety of pharmaceuticals has already been detected in wastewater effluents, surface waters and ground waters in the range of ng L^{-1} to a few µg L^{-1} (Halling-Sørensen et al. 1998; Fent et al. 2006), adding up to more than 100 different substances in the environment so far (Monteiro and Boxall 2010). Some highly consumed pharmaceuticals were even spotted in drinking water, e.g. diclofenac (DCF) (up to 6 ng L^{-1}) and ibuprofen (3 ng L^{-1}) (Jones et al. 2005). An increasing, often uncontrolled overuse of pharmaceuticals is one main explanation and the consumption of pain killers such as DCF is predicted to further grow (as reviewed by Schröder et al. 2016). Furthermore, many human pharmaceuticals are metabolized only partly or excreted largely unmodified from the ingested drugs and hence low levels of the active compounds are continuously discharged. On a global scale, sewage treatment plants (STPs) are the major route of the release of pharmaceuticals into aquatic ecosystems (e.g. Kolpin et al. 2002, US; Andreozzi et al. 2003, Europe; Ashton et al. 2004, UK) (Figure 1). Unfortunately, conventional technologies in STPs often do not

sufficiently remove these compounds that are mainly excreted by urine or faeces (Ternes 1998; Daughton and Ternes 1999; Ferrari et al. 2003). Additionally, veterinary and even human pharmaceuticals can be discharged due to the distribution of liquid manure and sewage sludge on agricultural areas and end up in the ground water, too. There is a compulsive assessment of the environmentally relevant characteristics for newly introduced veterinary drugs since 1994 and for human ones since 1998. But foodstuff often contains additives such as antibiotics that are classified as growth promoters and therefore are subjected to the 'Foodstuffs, consumer goods and animal feed' code and not mandatory pass through ecotoxicological testing (Fent 2003).

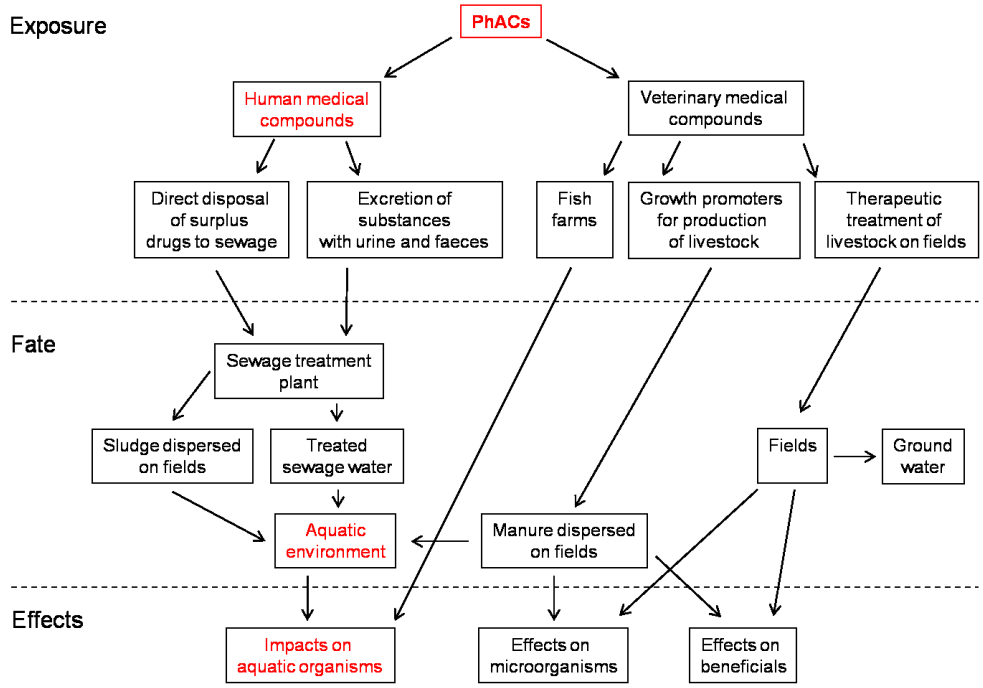


Figure 1. Anticipated exposure routes of pharmaceutically active compounds (PhACs) in the environment (modified from Halling-Sørensen et al. 1998). Highlighted in red is the approach of this work.

Pharmaceuticals are developed to modify certain physiological processes in humans but are not restricted to that (Halling-Sørensen et al. 1998). The occurrence of pharmaceuticals in aquatic ecosystems is well documented (Huschek et al. 2004; Fent et al. 2006) but little is known about their effects on non-target species in the environment (Fent et al. 2006, Ericson et al. 2010). According to Han et al. (2010), most investigations on pharmaceutical toxicity to non-target species are limited to lethal effects during acute exposure. Acute toxicities of selected human pharmaceuticals were determined using standardized tests with bacteria, algae

and crustaceans (Farré et al. 2001; Ferrari et al. 2003; Cleuvers 2004) or some fish species, mainly Japanese medaka (*Oryzias latipes*) and rainbow trout (*Oncorhynchus mykiss*) (Fent et al. 2006). Halling-Sørensen et al. (1998) stated almost two decades ago that many pharmaceuticals do not possess an acute aquatic toxicity due to low actual concentration ranges in the environment but significant cumulative effects on the metabolism of non-target organisms occur. Nonetheless, chronic toxicity tests are limited, maybe due to the complex experimental work (Santos et al. 2010). In general, acute toxicity challenges use orders of magnitude higher concentrations compared to chronic studies. Triebskorn et al. (2004, 2007) found the lowest observed effect concentration (LOEC) of different pharmaceuticals in rainbow trout to be 1 $\mu g\ L^{-1}$, and therefore in the environmentally relevant range. Effective concentrations based on acute toxicity tests range around 100 $mg\ L^{-1}$ (Fent et al. 2006). Therefore, the scientific discussion emphasizes the risk of misinterpretations of physiological impacts on aquatic organisms based on acute toxicity testing and stresses out the urgent need of chronic exposure studies (Hoeger et al. 2005; Fent et al. 2006; Triebskorn et al. 2007), hardly implemented until today. The topicality even increased recently, since three pharmaceuticals, including DCF, were added to the EU's pollutant 'watch list' of emerging aquatic pollutants of the Water Framework Directive (Decision 2015/495). Data on possible adverse effects in the aquatic biota, especially fish, is needed as a basis for a refined risk assessment.

Potential long-term ecotoxicological effects can be the accumulation of compounds in the aquatic organism (bioaccumulation), the promotion of antibiotic resistances, gene activation and silencing, changes of the immunological status and disturbances in the hormonal homeostasis (endocrine disruption). Ethinylestradiol (EE2), the potent synthetic estrogen used in contraceptive pills, is one of the most popular and alarming examples of pharmaceuticals in the aquatic environment. It is likely to cause feminization or even more harassing, seems to be responsible for the collapse of a fathead minnow (*Pimephales promelas*) population in a lake experimentally enriched with that substance (Kidd et al. 2007).

Endocrine changes of normal (fish) physiology due to endocrine disrupting chemicals (EDCs) are a great concern in ecotoxicology. EDCs are defined as 'exogenous substances or mixtures that alter function(s) of the endocrine system and consequently cause adverse health effects in an intact organism or its progeny or (sub)populations' (WHO 2002). These substances can be of natural or synthetic (man-made) origin (Reif et al. 2010). Since hormones control a number

of physiological functions such as development, growth, metabolism, reproduction and behavior (Norris and Carr 2013), disruptions of this system can cause severe changes. The mode of actions of EDCs are as diverse as the compounds themselves and include effects on hormone biosynthesis, release into circulation, blood plasma transportation as well as metabolism and excretion (Tyler et al. 1998; Vos et al. 2000; Sanderson 2006; Cheshenko et al. 2008). Additionally, some EDCs disrupt hormonal signaling by mimicking, blocking or modulating the interactions of endogenous hormones with their receptors (Sonnenschein and Soto 1998; Zoeller 2005; Tilghman et al. 2010). Concerning EDCs, alterations of the reproductive systems with emphasis on (anti)estrogenic and (anti)androgenic modes of actions are studied most intense so far (e.g. Jobling et al. 1998, 2006, 2009). Nonetheless, changes in the stress hormone system (Pottinger 2003), thyroid system (Jugan et al. 2010; Lorenz et al. 2011), immune system (Chalubinski and Kowalski 2006; Inadera 2006) as well as obesity and metabolic disorders (Desvergne et al. 2009; Gruen and Blumberg 2006) are under investigation.

1.2 Test substances

Overall, the diversity of therapeutic compounds is huge, but five major groups were defined (Heberer 2002): i) analgesics and anti-inflammatory drugs, ii) antibiotics, iii) antiepileptic drugs, iv) ß-blockers and v) blood lipid regulators. Non-steroidal anti-inflammatory drugs (NSAIDs) and ß-blockers are among the most frequently detected pharmaceuticals in aquatic ecosystems (Jones et al. 2002; Huschek et al. 2004) since they belong to the most prescribed classes of pharmaceuticals. They are continuously consumed and furthermore often resistant to biodegradation (Ternes 1998; Heberer 2002; Andreozzi et al. 2003). DCF and metoprolol (MTP), the two model substances used in the experiments presented, belong to these two groups of pharmaceuticals.

1.2.1 Diclofenac

Diclofenac (DCF, Figure 2) is a NSAID used to treat inflammatory reaction and pain such as back pain, migraine or pain due to fever in human and veterinary medicine (Zhang et al. 2008). Like acetylsalicylic acid or ibuprofen, DCF is sold over the counter. It is applied worldwide but records for many developing countries are unclear since no consistent data recording exists. In Germany, annual DCF consumption is around 90 t per year. Therefore, DCF belongs to the 15 most often sold drugs (Huschek et al. 2004). Estimates predict that 70 % of the consumed DCF are excreted without any modification via urine into the water

cycle, which accounts for 63 t annually (Meißner 2008). DCF is very persistent in the aquatic environment and shown to be incompletely subjected to catabolism during the passage through conventional STPs. Therefore, DCF was detected in concentrations of ng L^{-1} to low µg L^{-1} in sewage waters and consequently in surface waters around the world (e.g. Ternes 1998; Stumpf et al. 1999; Heberer 2002; Ashton et al. 2004; Gómez et al. 2007; Letzel et al. 2009). Stumpf et al. (1999) published the first record of DCF in the aquatic environment in 1999 and a few years later, DCF and ibuprofen were detected in drinking water in Germany at low ng L^{-1} concentrations (Heberer 2002). In Italy, about 5 µg L^{-1} were measured in wastewater effluents (Andreozzi et al. 2003).

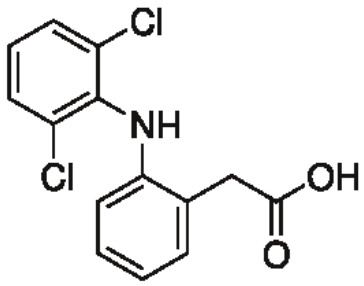


Figure 2. Chemical structure of DCF.

In humans, DCF inhibits the cyclooxygenases (COX-1 und COX-2), enzymes generally conserved across vertebrates (Grosser et al. 2002). COX enzymes catalyze the production of inflammatory prostaglandins and if they are inhibited, no such compounds are produced. Prostaglandins regulate a large number of physiological processes ranging from thermoregulation, water balance, glomerular filtration and homeostasis to control of ovulation (Fujimori et al. 2011). In fish, prostaglandins are essential for the reproduction, since they can influence the ovulation and spawning behavior as well as the oocyte maturation (Goetz et al. 1989). The prostaglandin E_2 (PGE_2) is involved in fish ovulation and might be regulated by COX-2 (Fujimori et al. 2011). Therefore, NSAIDs might cause reproductive impairments by disrupting the production of PGE_2 due to COX-2 inhibition. Furthermore, Díaz-Cruz et al. (2005) showed in their study about breast cancer in humans that cyclooxygenase inhibitors lead to a decreased expression of aromatase mRNA. Aromatase is the key enzyme for the conversion of testosterone to 17ß-estradiol (E2). The latter is essential for the gonadal differentiation and therefore sexual differentiation in fish (D'Cotta et al. 2001). Al-Qutob and Nashashibi (2009) demonstrated that feeding early developmental stages of Nile tilapia (*Oreochromis niloticus*) with DCF containing feedstuff lead to a later impairment of their

reproduction. Therefore, DCF might have modulatory effects on the regulation of reproduction in Nile tilapia.

In different studies negative impacts of low but environmentally relevant DCF concentrations on the physiology of rainbow trout and Japanese medaka were demonstrated (Schwaiger et al. 2004; Triebskorn et al. 2004, 2007; Hong et al. 2007; Cuklev et al. 2011; Lee et al. 2011). The LOEC of DCF in fish was set to be 1 µg L^{-1} (Triebskorn et al. 2004), leading to increasing public concern. Most studies were conducted with trout species since they are easy to maintain under experimental conditions and occur in freshwater and seawater environments. The authors showed that the exposure to concentrations as low as 1 µg L^{-1} DCF caused pathological changes in the liver tissue in rainbow trout and at 5 µg L^{-1} DCF severe morphological changes in the tissues of kidney and gills appeared. Memmert et al. (2013) conducted one of the few studies combining population relevant endpoints and histopathological effects in rainbow trout (*Oncorhynchus mykiss*) and zebrafish (*Danio rerio*) after DCF exposure. The authors concluded that DCF has most likely no adverse effect up to 320 µg L^{-1} DCF. In fact, a significant decrease in survival was found in early life stages of carp (*Cyprinus carpio*) only after exposure to 3 mg L^{-1} DCF for 30 days (Stepanova et al. 2013). Nonetheless, pharmaceuticals have the potential to act as endocrine disrupting compounds (EDC) (Kloas et al. 2009; Massarsky et al. 2011) and it was shown recently that DCF has a potential estrogenic endocrine activity already at environmentally relevant concentrations in fish hepatocytes *in vitro* (Gröner et al. 2015) and *in vivo* (Hong et al. 2007) as well as in amphibians (Efosa in prep., pers. comm.).

1.2.2 Metoprolol

Metoprolol (MTP, Figure 3) belongs to the therapeutic class of ß-adrenergic receptor antagonists, shortly ß-blockers. It binds to the ß-adrenoreceptors (ß-ARs) and blocks their actions. ARs are a class of G protein-coupled receptors building the adrenergic system. This regulates many aspects of vertebrate metabolism and function. Endogenous catecholamine hormones, epinephrine and norepinephrine, are the messengers and their binding stimulates the sympathetic nervous system, helping the organism to respond to physical, environmental and behavioral stressors (Wendelaar-Bonga 1997). Norepinephrine mainly acts as a neural transmitter substance with a localized effect while epinephrine possess systemic effects leading to an increased performance. Two AR types with different subtypes are distinguished. Briefly, the α-ARs with the main subtypes α_1 and α_2 use calcium (Ca^{2+}) as a second

messenger. The α_1-receptor regulates the muscle cell contraction and the α_2-type in the noradrenergic neurons the inhibition of transmitter release. Binding of epinephrine elevates the cytosolic concentration of cyclic adenosine monophosphate (cAMP) by activating the adenylyl cyclase, an enzyme converting ATP to cAMP (Campbell and Reece 2002). The cAMP then activates another protein, usually the protein kinase A and it is the main second messenger of the ß-ARs. Three different subtypes are commonly distinguished. The $ß_1$-typ is found in the heart and kidney tissue. It is influencing the heart rate, contractility and stimulus conduction in the heart and the release of the enzyme renin in the kidneys. The $ß_2$-typ mainly occurs in the liver, stimulating glycogenolysis. The $ß_3$-typ is located in the brown adipose tissue and it is associated with lipolysis and thermogenesis (Held et al. 2013).

MTP binds specific to $ß_1$-receptors and is therefore used to treat cardiovascular diseases like hypertension, heart arrhythmia or heart attack (Regårdh et al. 1974). In Germany, more than 90 t are sold annually and therefore MTP is, like DCF, one of the top-selling drugs (Huschek et al. 2004). MTP was detected in surface waters in concentrations up to 2.2 $\mu g\, L^{-1}$ (Ternes 1998). Information about the degradation rates of ß-blockers in STPs vary between 0-96 % (Ternes 1998; Andreozzi et al. 2003).

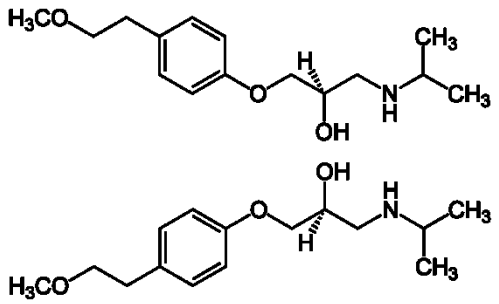


Figure 3. Chemical structure of MTP.

The adrenergic system is well-conserved in all vertebrates, including fish. Massarsky et al. (2011) could show that binding kinetics of fish and mammalian ß-ARs are quite similar and concluded that drugs affecting mammalian ARs will also affect fish species. According to Santos et al. (2010), ß-ARs can be found in the heart, liver and gonads of fish and hence there is a great probability that chronic exposures to ß-blockers lead to negative impacts in these non-target organisms. So far, only little information about the effects of MTP on the physiology of aquatic organisms is published (Liu and Williams 2007). Triebskorn et al.

(2007) conducted one of the rare studies on the effects of MTP on fish. They showed that concentrations as low as 1 µg L^{-1} affected the liver, but also kidney and gills of rainbow trout in various ways. The LOEC for damages to liver cells was found to be 1 µg L^{-1} and to gills to be 20 µg L^{-1}. Owen et al. (2007) could show that ß-blockers influence the development and reproduction in fish. They can lead to a delay in the embryonic development and evoke changes in plasma levels of sex hormones. Huggett et al. (2002) showed that MTP caused a decrease in testosterone in male medaka and a significant increase in E2 in both sexes. They supposed an increase in aromatase activity as a possible reason but did not verify it.

1.3 Fish as model organism in ecotoxicology

Fish, like all aquatic organisms, are exposed to numerous pollutants present in the aquatic environment throughout their lifetime. As fish share many physiological functions with humans, they also have many drug targets in common (Gunnarsson et al. 2008) and even common detoxification pathways may occur. Therefore, fish is a useful model organism to study the effects of human pharmaceuticals and their metabolites in surface waters. In contrast, aquatic invertebrates and plants lack many of these common drug targets. Nonetheless, many standard risk assessments are performed with crustaceans and algae but the transferability of these test results to predict effects in fish is rather questionable. For example, data on effects of estrogenic substances from invertebrates, that lack an estrogen receptor, cannot be used to predict estrogenic effects in fish which possess estrogen receptors (Gunnarsson et al. 2012). Furthermore, fish are a taxonomic diverse group with around 32,000 extant species (www.fishbase.org) adapted to different environmental conditions. Hence, extrapolations between fish species should be based on species-specific molecular knowledge (Celander 2011).

1.4 Physiological responses

Physiology is the study of the functions of an organism (Campbell and Reece 2002). As a consequence of a changing environment, organisms are forced to adapt to new conditions permanently. Three steps are usually involved in the physiological response of an organism to environmental conditions: (i) signal reception, (ii) signal transduction and (iii) (cellular) response. In the last stage, the signal triggers a specific cellular activity such as catalysis by an enzyme or activation of specific genes. Overall, this helps to ensure that crucial activities occur in the right cells, at the right time, and are well coordinated with the other cells of the organism (Campbell and Reece 2002), resulting in a proper adaptation to the outer conditions.

Physiological responses can be measured at different levels. Taking growth as an example, this can be measured directly by evaluating length and weight or rather indirectly by using specific biomarkers indicating growth. These in turn can be evaluated on the protein level in serum or at the gene expression level in their main synthesizing tissues.

1.4.1 Growth regulation

Growth in fish, like in all vertebrates, is under genetic control but also influenced by environmental factors such as temperature and food availability. These external stimuli and internal physiological conditions are merged and processed in the brain via the hypothalamus-pituitary axis and then further relayed to the target organs (Figure 4). The hypothalamus produces antagonistic polypeptides, growth hormone-releasing hormone and growth hormone-inhibiting hormone or somatostatin. The pituitary then produces growth hormone (GH) regulated by the hypothalamic hormones. GH binds to its receptors in the target organs, mainly the liver. There it stimulates the synthesis and release of the insulin-like growth factor I (IGF-I). Both, GH and IGF-I are transported bound to specific binding proteins in the blood circulation. IGF-I is involved in almost all processes responsible for somatic growth, including regulation of protein, lipid, carbohydrate and mineral metabolism in the cells as well as cell differentiation and proliferation. All these functions are mediated by IGF receptors. Besides the liver as the main producer for IGF-I, a variety of other tissues such as brain, muscle, kidney and gut produce IGF-I locally (Moriyama et al. 2000).

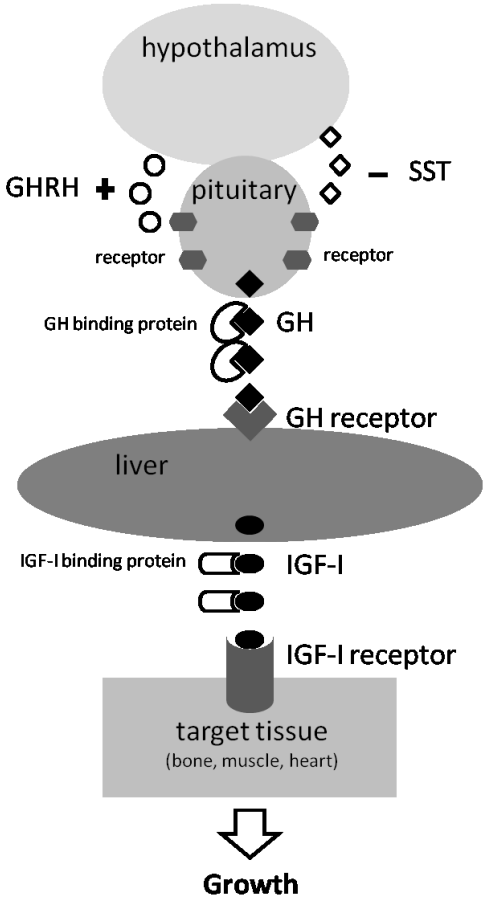


Figure 4. Schematic diagram of the endocrine regulation of growth in vertebrates, including fish. Growth hormone releasing hormone (GHRH) stimulates (+), while somatostatin (SST) inhibits (-) the release of hypophyseal growth hormone (GH). Attached to GH binding protein, GH circulates in the blood stream to the liver and induces the synthesis and release of insulin-like growth factor I (IGF-I), the main stimulator of somatic growth in target tissues (modified after Moriyama et al. 2000).

1.4.2 Detoxification mechanisms

The occurrence of xenobiotics, i.e. foreign substances, in aquatic ecosystems is especially important since the affected organisms cannot escape from the continuous exposure. In fish, two pathways exist for xenobiotics to enter the body. Waterborne chemicals can directly enter the fish via its openings and/or contaminated food can be ingested (Streit 1998). Different responses to xenobiotics evolved related to the chemical nature of the compounds and scientists use them as biomarkers for early detection and assessment of impacts of pollutants. They developed such biomarkers as a tool to study pollution-induced variations in biological systems (Peakall 1992), which are often related to detoxification processes. Their production is regulated by receptors that can recognize xenobiotics and different types of proteins work together. This increases the ability to excrete the xenobiotics and prevent harmful

accumulation in the body. The main target organ is the liver, where xenobiotics but also endogenous compounds such as steroid hormones are metabolized (Waxman et al. 1988, Parkinson 1996). Some exogenous chemicals are excreted largely unmodified but the majority is detoxified during a biotransformation process (Figure 5). Biotransformation is defined as the transformation of chemical compounds in an organisms (Parkinson 1996). The biotransformation processes consist of a range of proteins and are characterized by different phases. During phase I and II, biotransformation enzymes are active, while efflux pumps are working in phase 0 or III (Xu et al. 2005). Overall, fat-soluble substances are metabolized to more water-soluble compounds that can be excreted more easily from the body.

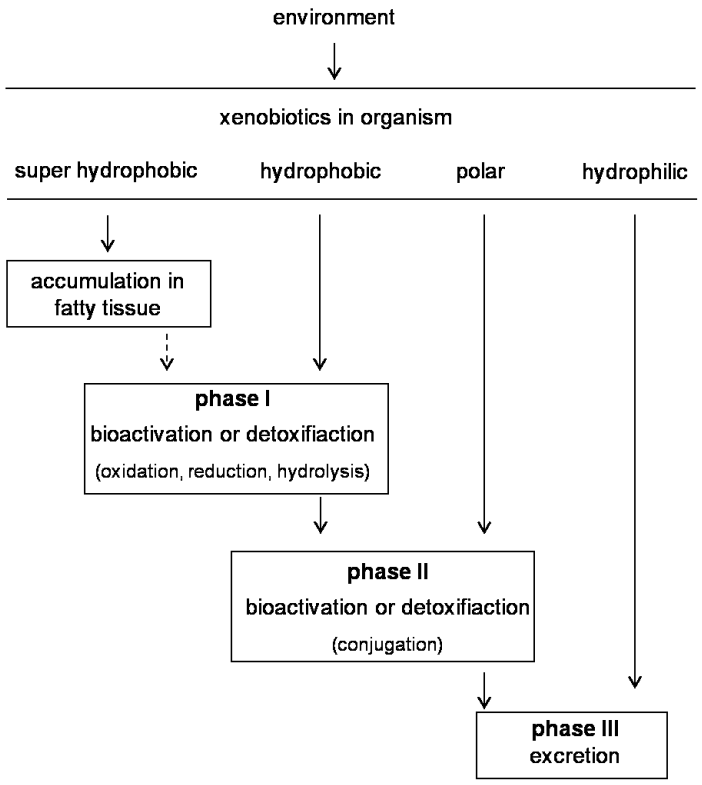


Figure 5. Pathways of biotransformation of xenobiotics in fish (modified from van Leeuwen and Hermens 1995).

In more detail (Figure 6), during phase I oxidation occurs to increase the polarity and therefore the water solubility of the xenobiotics, mainly catalyzed by the cytochrome P450 (CYP) system. This system is essential for the biotransformation of contaminants and the CYP1A subfamily is the most important one with regard to biotransformation and bioactivation of xenobiotics. In general, many xenobiotics induce the *de novo* synthesis of cytochrome P450 proteins in fish. The enzymes metabolize, among others, exogenous

compounds such as food additives and drugs. They are mainly found in the liver but various other tissues such as kidney, heart and gills exhibit them too (Goksøyr and Husøy 1998). CYP1A is known to be involved in the metabolism of many environmental contaminants in fish (e.g. polycyclic aromatic hydrocarbons; van der Oost et al. 2003) but exposure to a range of pharmaceuticals (including clofibrate, DCF and carbamazepine) often revealed CYP1A inhibition or no effect (e.g. Laville et al. 2004; Thibaut et al. 2006). Only few studies could demonstrate an induction of CYP1A activity (e.g. Laville et al. 2004; Hong et al. 2007; Gröner et al. 2015). Subsequently, during phase II, conjugation of the compounds with either glutathione, sulfate, amino acids or glucuronic acid occurs (Goodman Gilman et al. 1990). These reactions are catalyzed by different transferases. Electrophilic compounds are conjugated by the glutathione S-transferase (GST) and nucleophilic ones by the glucuronyl transferase. Water solubility and excretability further increase. Finally, the modified compounds are transported and excreted by efflux pumps. These are membrane proteins, actively transporting a wide range of compounds out of the cells. They belong to the large superfamily of ATP-binding cassette (ABC) proteins (Leslie et al. 2005). Their prominent members are the permeability glycoprotein (P-gp) and the related multidrug resistance protein (MDRP). The gene coding for P-gp is denoted ABCB1 and for MDRP is ABCC. P-gps are involved in the transport of un-metabolized xenobiotics in phase 0, while MDRPs protect tissues from xenobiotic-induced damage by transporting mainly conjugated metabolites (phase III) (Leslie et al. 2001). Fish excrete via bile, urine and gills (Nimmo 1987; Clarke et al. 1991). It has been suggested that phase I-III enzymes are regulated in a coordinated way, resulting in a high protection level of cells from xenobiotic damage (Bard 2000; Leslie et al. 2005).

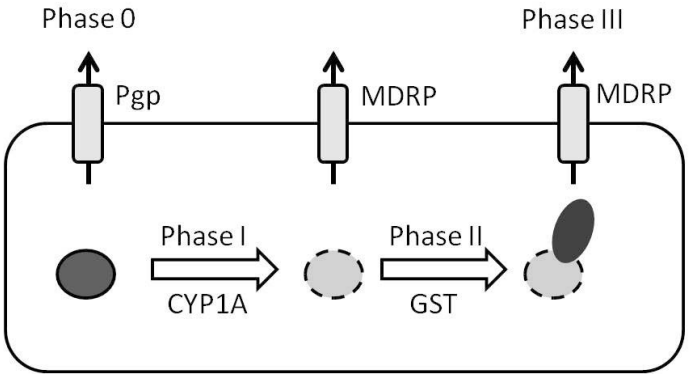


Figure 6. Biotransformation pathway in a liver cell. CYP1A: cytochrome P4501A monooxygenase, GST: glutathione-S-transferase, MDRP: multidrug resistance protein, Pgp: permeability glycoprotein.

1.4.3 Oxidative stress

Oxidative stress is defined as a disruption of the pro-antioxidative balance, meaning the generation and removal of radical species within an organism is imbalanced. These radical species mainly involve oxygen and are referred to as reactive oxygen species (ROS). ROS overwhelm the cellular defenses and damage proteins, membranes and DNA (Kelly et al. 1998). Many xenobiotics likely enhance the production of ROS and therefore lead to oxidative damage (Winston and Di Giulio 1991; Oruç 2010). Different antioxidative enzymes for the detoxification of ROS exist in all organisms. The most important enzymes are superoxide dismutase, catalase (CAT) and glutathione peroxidase. Changes in their activity are often used as biomarkers of pollutant-mediated oxidative stress (Winston and Di Giulio 1991). Another mean of measuring the toxic consequences of oxidative stress is to evaluate the effects at the subcellular level including lipid peroxidation (LPO). LPO is probably the most extensively studied process induced by free radicals (De Zwart et al. 1999). A direct measurement of the endogenous primary LPO products is difficult and therefore secondary oxidation products are analyzed. The most often used assay for LPO is the thiobarbituric acid reactive substance (TBARS) test. During this test, a colored adduct from the reaction of LPO products and thiobarbituric acid (TBA), mainly malondialdehyde (MDA), is produced. Since TBA also reacts with other oxidation products the test is nonspecific and only a rough measure (Kelly et al. 1998).

1.4.4 Regulation of reproduction

Similar to growth regulations, reproduction in all vertebrates is under endocrine control. Internal (e.g. nutritional status) and external stimuli (e.g. photoperiod and temperature), influencing the central nervous system, are processed by the hypothalamus-pituitary gonad (HPG) axis, regulating reproduction (Norris and Carr 2013).

Briefly, gonadotropin-releasing hormones (GnRHs) are the main neuropeptides produced by neuroendocrine cells of the hypothalamus stimulating the synthesis and secretion of the pituitary gonadotropins, namely luteinizing hormone (LH) and follicle stimulating hormone (FSH). LH and FSH regulate cell differentiation, proliferation and steroidogenesis of gonad tissue (Lubzens et al. 2010; Schulz et al. 2010), leading to the production and release of sex steroids (androgens, estrogens and progesterone). Finally, the sex steroids affect target cells and cause feedback mechanisms on hypothalamus and pituitary (Figure 7).

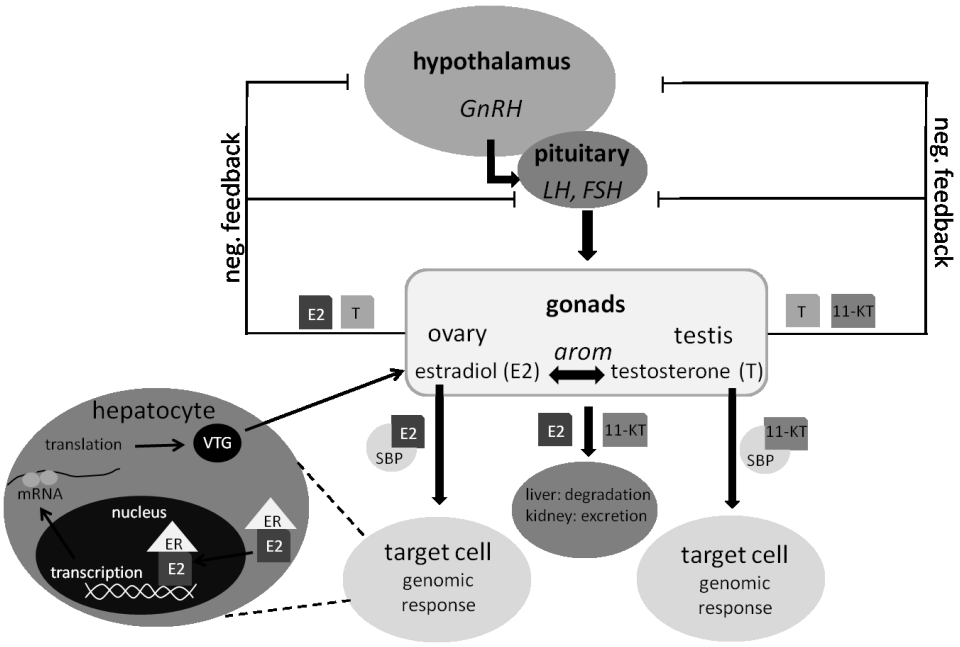


Figure 7. Schematic diagram of endocrine regulation of sex steroids in fish by the hypothalamus-pituitary-gonad (HPG) axis. Hypothalamic gonadotropin-releasing hormone (GnRH) stimulates the synthesis and release of pituitary gonadotropins, namely luteinizing hormone (LH) and follicle stimulating hormone (FSH). The gonadotropins stimulate the synthesis and release of sex steroids in the gonads and these can cause negative feedbacks on hypothalamus and pituitary. Circulating sex steroids (estradiol: E2; testosterone: T; 11-ketotestosterone: 11-KT) affect target cells. Shown is for instance the effect of E2 in liver cells, inducing vitellogenin (VTG) synthesis and influencing sex differentiation and egg maturation. arom: aromatase (catalyzes the conversion of T into E2); ER: estrogen receptor; SBP: sex hormone binding proteins.

LH and FSH consist of two subunits, a common glycoprotein-hormone α-subunit and a specific β-subunit and their biological activity is mediated by a membrane-bound G-protein coupled receptor (Levavi-Sivan et al. 2010). While FSH most likely stimulates early development of the ovarian follicle and spermatogenesis in the testis, LH is influencing the final gamete maturation leading to ovulation in females and spermiation in males (Levavi-Sivan et al. 2010; Lubzens et al. 2010; Schulz et al. 2010). Gonadotropins are key hormones of the reproduction but their effects are rather indirect via stimulation of sex steroid synthesis and local growth factors in the gonads (Kloas et al. 2009; Lubzens et al. 2010; Schulz et al. 2010). Sex steroids are essentially important for different reproductive processes including vitellogenesis (Lubzens et al. 2010), spermatogenesis (Schulz et al. 2010), final maturation of gametes and ovulation as well as spermiation (Nagahama and Yamashita 2008; Scott et al. 2010), expression of secondary sexual characteristics (Borg 1994) and reproductive behavior (Munakata and Kobayashi 2010). Furthermore, they modulate physiological functions such as

metabolism (Mauvais-Jarvis 2011) and the immune system (Vainikka et al. 2004; Gilliver 2010). In fish, the predominant androgen is 11-ketotesterone, while testosterone and dihydrotestosterone can be found in all higher vertebrates (Tinsley and Kobel 1996; Kloas et al. 2009). Two main signal transduction pathways are mediating the actions of sex steroids. They act via nuclear receptors directly activating transcriptional cascades (Aranda and Pascual 2001; Björnström and Sjöberg 2005) or they bind to membrane-associated steroid receptors, initiating non-genomic steroid actions (Thomas et al. 2006; Hammes and Levin 2007).

Sex steroids regulate gonadotropin synthesis and secretion via feedback mechanisms on the hypothalamus and pituitary (Zohar et al. 2010), leading to permanent adjustments of the activity of the HPG axis and maintenance of the homeostasis of the organism (Kloas and Lutz 2006). Positive and negative feedback effects of sex steroids on the synthesis and release of LH and FSH in fish have been reported (e.g. Huggard-Nelson et al. 2002).

1.5 The model species *Oreochromis niloticus*

O. niloticus (Nile tilapia, Figure 8) is a teleost fish belonging to the order of *Perciformes* (perch-like) and the family of C*ichlidae* (cichlids). It is native to tropical and subtropical Africa and widely distributed in the Nile and Niger river basin. *O. niloticus* is living preferable in shallow waters at temperatures of 31-36 °C, but can withstand a huge temperature range of 12 - 42 °C. It is an omnivorous grazer and relatively resistant to poor water quality, indicated by its occurrence in sewage canals or irrigation channels (Bailey 1994). Sexual maturity is reached at three to six months depending on the temperature. In general, reproduction occurs at temperatures higher than 20 °C. Breeding is feasible all year around (asynchronous breeder) and the females incubate the eggs in their mouth and brood the fry after hatching until the yolk sac is absorbed. This is called maternal mouth breeding (FAO 2016).

Figure 8. *Oreochromis niloticus*, Nile Tilapia.

Tilapia (including all species) is the second most important group of farmed fish and therefore of high economical value (Figure 9, FAO 2016). It is frequently cultured in net cages of surface waters with great potential to be contaminated by environmental pollutants, including pharmaceuticals such as DCF. The main producer countries are China and Egypt, followed by Indonesia, Thailand and Brazil, countries all having hardly any wastewater treatment and only insufficient environmental regulations. Since Nile tilapia is easy to grow all year round it is repeatedly used in toxicological research (Costa et al. 2012).

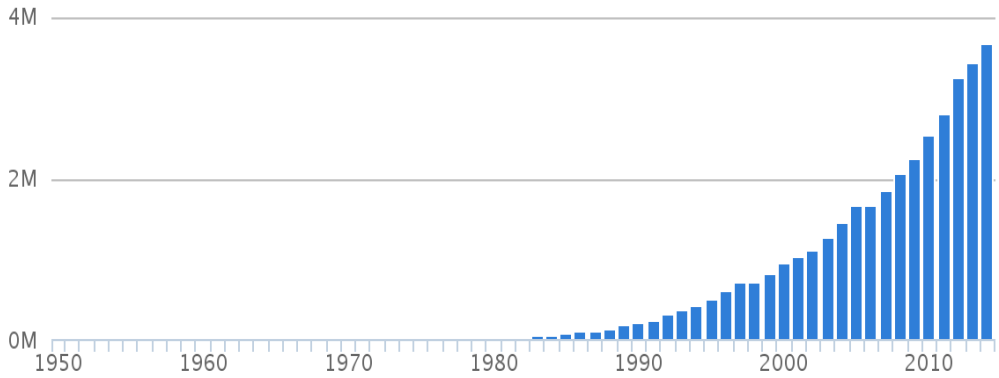


Figure 9. Global aquaculture production of *Oreochromis niloticus* (tones) (1950-2015). Source: FAO Fish Stat.

1.6 Aim of the study

The occurrence of human pharmaceuticals in aquatic ecosystems has been well documented in recent years. However, little is known about ecotoxicological effects due to chronic exposure on non-target organisms such as fish. These are particularly important, since fish cannot escape from the continuous exposure. Aim of the study was to investigate the effects of two commonly found substances belonging to two different therapeutic classes of drugs, DCF and MTP, on Nile tilapia (*O. niloticus*). Exposure concentrations ranged from environmentally relevant to 100-fold thereof. Nile tilapia is one of the most important aquaculture fish species worldwide. The cultivation of aquaculture fish in contaminated water is likely to occur in many tropical countries where open aquaculture systems (net cages) are used. Overall, the ability of DCF and MTP to alter the expression of selected genes in the liver was investigates *in vitro* and *in vivo*. Additionally, population relevant responses

integrated with molecular biological and histological endpoints were assessed in two identically designed long-term exposure experiments (80 days post-hatch) *in vivo*.

The following working hypotheses were established:

In vitro exposure of *O. niloticus* to DCF or MTP in the range of $4x10^{-9}$ M to $4x10^{-7}$ M (environmentally relevant up to 100-fold higher)

(1) affects detoxification mechanisms in liver cells by inducing biotransformation phase I to III enzymes, indicated by an increase in gene expression of CYP1A, GST and MDRP.
(2) induces vitellogenin gene expression, indicating the potential estrogenic mode of action.

In vivo exposure of *O. niloticus* to DCF or MTP in the range of $4x10^{-10}$ M to $4x10^{-7}$ M (environmentally relevant up to 100-fold higher)

(3) has most likely no negative effects on the general parameters hatching and survival but adverse impacts on growth parameters, including altered gene expression of hypophyseal GH and hepatic IGF-I.
(4) causes adverse alterations of the gills, one of the first barrier organ in exposed fish, expressed by histopathological changes.
(5) affects biomarkers associated with endocrine activity concerning gonadotropin gene expression (LH, FSH) and estrogenicity (VTG). The potential estrogenic mode of action is indicated by an increase of VTG gene expression.
(6) affects detoxification mechanisms in liver and gill tissue by inducing biotransformation phase I to III enzymes, indicated by an increase in gene expression of CYP1A, GST and MDRP.
(7) is likely to cause oxidative stress in various organs of fish based on elevated TBARS levels.

2 Materials and methods

2.1 Chemicals

All chemicals were obtained from Sigma Aldrich (Steinheim, Germany) or as indicated otherwise. DCF sodium salt and MTP tartrate (Table 1) were dissolved in Milli-Q-grade water. EE2 (CAS: 57-63-6) with a purity of >98 % was dissolved in ethanol (p.a.) and substances were used for exposure stock preparation to reveal final test concentrations.

Table 1. Main physical-chemical properties of the tested substances.

	Diclofenac (sodium salt)	**Metoprolol (tartrate)**
therapeutic class	anti-inflammatory	ß-blocker
CAS number	15307-79-6	56392-17-7
molecular weight (g mol^{-1})	318.13	684.82
formula	$C_{14}H_{11}Cl_2NO_2 \cdot NaCl$	$(C_{15}H_{25}NO_3)_2 \cdot C_4H_6O_6$
purity	>98 %	>98 %
Log K_{ow}	0.57	1.69
solubility (mg L^{-1})	50,000	50,000

2.2 Experimental animals

2.2.1 *In vitro* experiments

Male Nile tilapia (*O. niloticus*), weighing between 100-200 g, were obtained from the stock of Leibniz-Institute of Freshwater Ecology and Inland Fisheries, Berlin, Germany. Prior to cell isolation, fish were kept in an aerated 800 L tank with water flow-through (26 ± 1 °C, natural photoperiod) for several weeks and fed daily *ad libitum* with a commercial fish food (Aller Aqua Primo, Golßen, Germany).

2.2.2 *In vivo* experiments

Fertilized eggs of *O. niloticus* were collected from the permanent brood stock of the Leibniz-Institute of Freshwater Ecology and Inland Fisheries. No acclimation of eggs was conducted. Eggs were placed directly into the experimental set-up to reflect natural conditions were eggs are immediately exposed to environmental pollutions after release. The experiments were run in accordance with the local animal welfare committee (LaGeSo G 0276/12).

2.3 Primary hepatocytes – *in vitro* experiments

2.3.1 Primary hepatocyte isolation

Hepatocytes were isolated according to the method described by Schmid et al. (2000) with slight modification. Briefly, fish were anesthetized by immersion in tricaine methanesulfonate (MS222; 8 g L^{-1}, Sigma, Germany) and injected with heparin (3000 U dissolved in 0.6 mL water; Roth, Karlsruhe, Germany). Perfusion of liver, carried out at room temperature, was performed retrogradly by inserting a catheter (1 mm diameter) via the *bulbus arteriosus* into the heart. First, calcium-magnesium free (CMF) medium (100 mL, Table 2) was used to wash out blood. Next, tissue was digested for approximately 20 min with calcium-magnesium containing (CMC) medium (50 mL, Table 2), supplemented with collagenase D and H (0.25 mg mL^{-1} each; Roche, Mannheim, Germany). The liver was then perfused with 50 mL CMF medium and removed from the body cavity. Finally, the liver was transferred into ice-cold CMF medium and to receive a dispersed cell solution minced with a scalpel. The cell suspension was filtered through meshes of 250 and 50 µm, centrifuged (4 °C, 70 *g* for 5 min, 50 *g* for 5 min, 30 *g* for 5 min) and the resulting cell pellet was resuspended in minimum essential medium (MEM, Table 2). Cell density was counted using a Neubauer chamber. Hepatocytes were seeded at a density of 1 x 10^6 cells mL^{-1} MEM in sterile culture discs (35 mm diameter) and incubated overnight at 20 °C under normal air conditions in an incubator (Heraeus, Hanau, Germany).

Table 2. Composition of media used for perfusion and culture of *Oreochromis niloticus* primary hepatocytes (according to Schmid et al. 2000). CMF: calcium-magnesium free medium, CMC: calcium-magnesium containing medium, MEM: minimal essential medium.

	CMF	**CMC**	**MEM**
NaCl (mM)	142	142	142
KCl (mM)	5.4	5.4	5.4
$CaCl_2$ (mM)	-	2.4	2.4
$MgSO_4*7H_2O$ (mM)	-	-	0.81
Na_2HPO_4 (mM)	0.42	0.42	0.42
KH_2PO_4 (mM)	0.44	0.44	0.44
$NaHCO_3$ (mM)	0.43	-	0.43
HEPES (mM)	15	15	20
amino acids (50x) (mL/L)	-	-	20
vitamins (100x) (mL/L)	-	-	20
NaEDTA (mM)	5	-	-
glutamine (mM)*	1	-	-

Media were adjusted to pH 7.5 and 1 mL per 100 ml medium of a penicillin-streptomycin solution (10,000 units penicillin and 10 mg streptomycin per mL) and glutamine* were added after sterile filtration and immediately before use. Amino acids, vitamins and glutamine were obtained from PAN Biotech (Aidenbach, Germany).

2.3.2 Hepatocyte treatment

Following overnight culture without exposure to chemicals, hepatocytes were exposed to test substances for 24 h (Table 3) for target gene transcription analyses. MEM was changed every 12 h and 2/3 of the culture medium including test substance concentrations were replaced twice. EE2 was included as a positive control for VTG induction (for concentrations, see Table 3); MEM-only was used as control. After 24 h of exposure, culture medium was removed and 700 µL Trizol (Invitrogen, Darmstadt, Germany) was added. Cells were harvested and immediately frozen in liquid nitrogen and stored at -80 °C until further analysis.

Table 3. Experimental design of *Oreochromis niloticus* primary hepatocyte *in vitro* exposure to DCF, MTP and ethinylestradiol (EE2).

	Control	**Diclofenac**			**Metoprolol**			**EE2**
[M]	0	$4x10^{-9}$	$4x10^{-8}$	$4x10^{-7}$	$4x10^{-9}$	$4x10^{-8}$	$4x10^{-7}$	10^{-6}
[µg L^{-1}]	0	1.27	12.72	127.24	1.07	10.69	106.94	0.3

2.4 Flow-through system – *in vivo* long-term exposure

2.4.1 Experimental set-up

Long-term exposure experiments were run in a temperature controlled continuous flow-through system (FTS) to maintain constant exposure conditions for the duration of the experiments. Two *in vivo* experiments were conducted, identical in experimental design, but with two different pharmaceuticals in the exposure medium, namely DCF and MTP.

The FTS consists of a water preparation unit providing artificial fresh water (AFW) and of a water bath equipped with a maximum of sixty-four 9 L glass aquaria (30 x 20 x 14.5 cm). Four glass aquaria are clustered (Figure 10), receiving exposure medium from a common mixing chamber. Using a peristaltic pump, stock solutions are continuously dosed into the mixing chambers with constant temperature controlled AFW inlet, regulated by a rotameter. The resulting exposure medium flows directly into the cluster of four replicate test aquaria per test concentration (140 mL min^{-1} ± 5 %). Stock solutions were renewed every fourth day and stored in light-protected glass bottles. The flow rate was set to at least 50 L exposure medium per tank and day, which means a water exchange rate of about seven tank volumes per day. AFW flow rates were measured once per week, stock solution flow rates were adjusted daily. Each tank was aerated and temperature was set to 27 ± 1 °C. The photoperiod was 12 h light to 12 h dark.

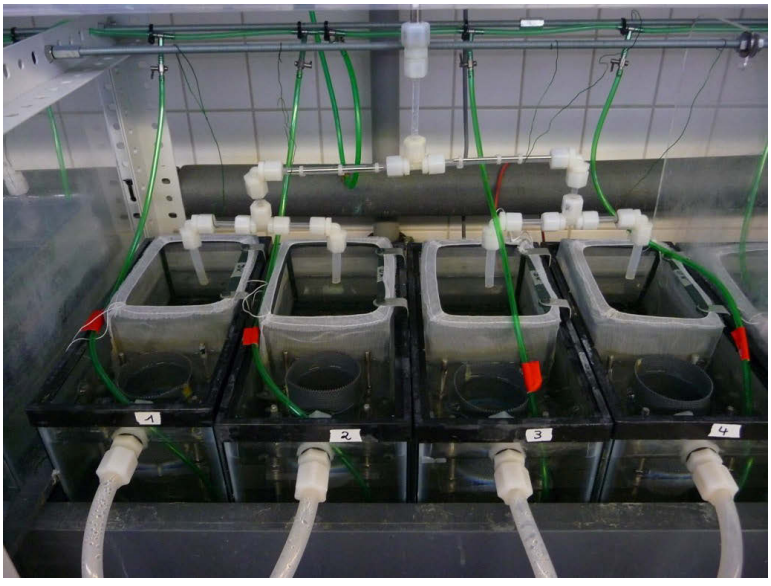


Figure 10. Experimental set-up in the flow-through system, showing one cluster (one treatment) including the different rearing devices.

AFW consists of analytical grade salts dissolved in Milli-Q- grade water. Reconstituted water then passed through several filters (5, 1, 0.5, 0.45 µm) to remove particulate material, a UV-sterilizer and a final particle filter (Lutz et al. 2008). Water temperature, pH and dissolved oxygen were recorded every working day using a portable multimeter (HQ40d multi, Hach Lange GmbH, Düsseldorf, Germany). Nutrients (NH_4^+, NO_2^-, NO_3^-, PO_4^{3-}) were measured once per week in one tank per cluster. To maintain water quality, tanks were cleaned regularly. Biofilm accumulating on the inner surfaces of the aquaria was scraped off and detritus was siphoned from the tanks.

The FTS was operated one week prior to egg transfer to insure substance concentrations of demand. Eggs (24 per tank) were carefully transferred into the glass aquaria filled with 7L of exposure medium. No acclimation phase was included into the experimental design to reflect natural conditions where eggs are immediately exposed to environmental pollution. Special incubation devices (Figure 11) were constructed providing permanent movement of the eggs by air bubbles, simulating the maternal care procedure. Therefore, a sieve was mounted on a vertically adjustable rack and an air stone was placed beyond each device to ensure slight movement of the eggs. After yolk sack adsorption free swimming larvae were released into a breeding container placed into the tank and when all larvae were free swimming and feeding, larvae were released into the entire glass aquarium. Stainless steel meshes kept juvenile fish back in the aquaria. Free-feeding fish were fed at least four times per day with a commercial dry food (Aller Artex).

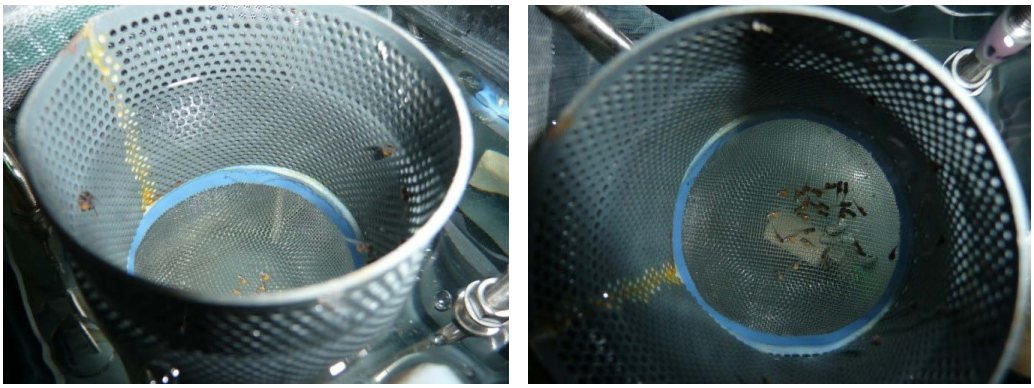


Figure 11. Breeding device. Nile tilapia are mouth breeders and this device fulfilled the tasks of maternal care, permanent movement of the eggs, by air bubbles to ensure constant supply of fresh water enriched with oxygen (for reason of visibility, the air stone was switched off to take these pictures).

Nominal test concentrations were measured weekly by quadrupole time-of-flight liquid chromatography/mass spectrometry (Q-TOF LC/MS) throughout the entire experiment (see 'DCF and MTP extraction and analyses' for details). Waste waters were disposed through an activated-charcoal filter in order to remove the exposure chemicals from the water to prevent for contaminations of the environment.

2.4.2 Experimental design

For both test substances, four nominal concentrations and a control group were included into the experimental design (Table 4). Test concentrations were chosen according to concentrations found in the environment, with the two lowest test concentrations approaching the environmental concentrations. The experiment was run for 80 days post-hatch (dph) and sampling was performed after 8, 30 and 80 dph. Depending on the fish size different parameters were recorded and analyzed (Table 5).

Table 4. Experimental design of long-term exposure experiments in the flow-through system.

	Diclofenac					**Metoprolol**				
[M]	0	$4x10^{-10}$	$4x10^{-9}$	$4x10^{-8}$	$4x10^{-7}$	0	$4x10^{-10}$	$4x10^{-9}$	$4x10^{-8}$	$4x10^{-7}$
[$\mu g\ L^{-1}$]	0	0. 13	1.27	12.72	127.24	0	0.11	1.07	10.69	106.94

Table 5. Summary of sampling and endpoints according to the developmental stages. dph: days post hatch, CYP1A: cytochrome P4501A monooxygenase, GST: glutathione-S-transferase, MDRP: multidrug resistance protein, LH: luteinizing hormone, FSH: follicle stimulating hormone, GH: growth hormone, IGF-I; insulin-like growth factor I, VTG: vitellogenin, TBARS: thiobarbituric acid substances.

Sampling points/ endpoints	prehatch	**8 dph (larvae)**	**30 dph (juvenile)**	**80 dph (adult)**
hatching rate	X			
survival		X	X	X
morphological				
length [mm]		X	X	X
wet weight [g]		X	X	X
gill histology				X
molecular biological				
CYP1A		X	X	X
GST		X	X	X
MDRP		X	X	X
LH				X
FSH				X
GH				X
IGF-I				X
VTG				X
TBARS				X

2.4.3 DCF and MTP extraction and analyses

Effective pharmaceutical concentrations were analyzed in duplicates weekly using Q-TOF LC/MS. Exposure medium samples (200 mL) were removed from one tank per cluster and concentrated using solid phase extraction with C18 cartridges. Cartridges were prepared by filling 500 mg octadecyl (Bakerbond, 40 µm Prep LC), enclosed by two PTFE frits (Bakerbond) into glass columns (Bakerbond spe 7328-06; J.T. Baker, Germany). Methanol (HPLC-MS grade) and formic acid (LC-MS grade) were obtained from Roth and Fluka, respectively. Cartridges were preconditioned and equilibrated with methanol and Milli-Q-water, 5 mL each. After passing the exposure media of each treatment, cartridges were dried under nitrogen. Elution of substances was achieved with methanol (3 x 2 mL). Afterwards samples were concentrated to dryness at 40 °C under nitrogen using a rotary evaporator (Büchi R-200 with heating bath B-490, Flawil, Switzerland) combined with a vacuum pump

(ILMVAC, Ilmenau, Germany). Samples were re-dissolved in 1 mL methanol. Control samples and samples of $4x10^{-10}$ M were measured directly; the others had to be diluted with methanol + 0.1 % formic acid ($4x10^{-9}$ M 1:5, $4x10^{-8}$ M 1:50, $4x10^{-7}$ M 1:500). DCF and MTP concentrations of water samples were determined by Q-TOF LC/MS. Details of the applied methods are provided in the annex.

2.4.4 Tissue sampling

For both *in vivo* experiments, all fish were sampled within one day at the time of sampling. Fish were anaesthetized using ice water bath and killed by spinal transection. Growth parameters of each individual fish were recorded. Different tissue samples were taken in the following order: first and second right gill arches were dissected and immediately frozen in liquid nitrogen. For gill histology the third left gill arches of experimental fish were dissected and transferred into embedding cassettes and fixed in Bouin´s solution (Sigma, Germany) for 24 h. Samples were transferred to EtOH (70 %), the EtOH solution was renewed two times (after 24 h) and storage was conducted in EtOH (70 %) until further processing.

Brain and pituitary samples were taken and immediately frozen in liquid nitrogen. Furthermore liver and muscle samples were taken and immediately frozen in liquid nitrogen as well. Frozen samples were stored at -80 °C until further processing.

2.4.5 General parameters

2.4.5.1 Hatching

Hatching of larvae was recorded daily. Percentage of hatching success was calculated by dividing the number of hatched larvae by the number of inserted eggs.

2.4.5.2 Growth

Fish total body length (tip of mouth to end of tail fin) and body wet weight were measured for every individual fish. Length was determined using a caliper rule to the nearest mm and wet weight to the nearest mg using a balance (Sartorius, Göttingen, Germany). Parameters were analyzed on a tank replicate basis to reduce the impact of individual outliers. The arithmetic mean and standard deviation of the mean values of the four replicates per treatment were calculated.

2.4.5.3 Morphological indices

Different morphological indices were calculated to have some indication about nutritional status (Futon´s condition index, CI) and degree of pollution (hepatosomatic index, HSI) of test organisms:

CI [g/cm³] = wet weight [g] x 100 / (length)^3 [cm] (according to Fulton 1904)

HSI [%] = (liver weight [g] / wet weight [g]) x 100

2.4.5.4 Survival

Mortality was recorded daily. Mortality rates were calculated by dividing the number of dead fish by the initial number of larvae hatched. Fish removed during sampling were not considered. Survival rates (%) were calculated by subtracting mortality rates from 100.

2.4.6 Gene expression analyses

Samples obtained from *in vitro* and *in vivo* experiments were subjected to gene expression analyses. Chemicals and reagents used for gene expression analyses are listed in Table 6.

Table 6. Chemicals and reagent kits used for gene expression analyses (listed in the order of usage).

Chemicals/reagent kits	**Details/description**	**Supplier**
2-mercaptoethanol	99 % p.a.	Roth, Karlsruhe, Germany
RNeasy Plus Micro kit	buffer RLT Plus (lysis buffer, contains a guanidine salt); Buffer RW1 (contains a guanidine salt and EtOH); Buffer RPE (washing buffer, concentrate); RNase free water; spin columns	Qiagen, Hilden, Germany
ethanol (EtOH)	>99 % p.a. or diluted with nuclease free water	Roth, Karlsruhe, Germany
nuclease free water	sterile, tested and certified to be free of DNase and RNase activity	VWR, Darmstadt, Germany
Trizol Reagent	contains phenol and guanidine isothiocyanate	Invitrogen, Darmstadt, Germany
chloroform	≥ 99 % p.a.	Roth, Karlsruhe, Germany
isopropanol	2-propanol, ≥ 99.8 % p.a.	Roth, Karlsruhe, Germany

Table 6. Continued.

RNA 6000 Nano kit	RNA Nano chips; RNA-ladder (0.2, 0.5, 1.0, 2.0, 4.0, 6.0 kb length); RNA marker; RNA dye concentrate (5x); gel matrix; spin filters	Agilent Technologies, Waldbronn, Germany
DNase I kit (amplification grade)	10x DNase I reaction buffer; DNase I enzyme (1U/µL); EDTA (25 mM)	Invitrogen, Darmstadt, Germany
AMV-RT kit	AMV-RT buffer (10x); AMV-RT enzyme (2500 U)	Biozym, Hess. Oldendorf, Germany
poly(dt) primer	PCR grade; 0.1 nM μL^{-1} stock solution; sequence: $CCTGAATTCTAGAGCTCA(T)_{17}$	Biometra, Göttingen, Germany
dNTPs	PCR grade, 10 mM each	Qiagen, Hilden, Germany
MMLV-RT kit	MMLV-RT buffer (10x); DTT (100 mM); MMLV-RT enzyme (200 U/µL)	Biozym, Hess. Oldendorf, Germany
platinum Taq DNA polymerase kit	10x PCR Buffer (-$MgCl_2$), $MgCl_2$ (50 mM), Platinum Taq Polymerase (5U/ µL)	Invitrogen, Darmstadt, Germany
SYBR Green solution	DNA dye, concentrate (10000x) in DMSO; used 1:200 diluted	Invitrogen, Darmstadt, Germany
agarose gel	Gel for electrophoresis, with ethidium bromide (10 mg/mL)	Invitrogen, Darmstadt, Germany
TAE-buffer	1x (pH: 8.5, 20 mM Tris–HCl, 0.1 % acetic acid (p.a.), and 1 mM NaEDTA)	Roth, Karlsruhe, Germany
bromphenolblue buffer	loading dye: 100 µL glycerin, 99 µL nuclease free water, 1 µL bromphenolblue	
QIAquick gel extraction kit	silica-membrane-based purification of DNA fragments from gels (cleanup of up to 10 µg DNA (70 bp to 10 kb)); PB buffer contains guanidine hydrochloride and 2-propanol, PE buffer (1x) diluted with EtOH (100 %) to (5x)	Qiagen, Hilden, Germany

2.4.6.1 RNA extraction

Depending on the amount of tissue available, the protocol of total RNA extraction varied. Extraction of total RNA from pituitary was carried out using the RNeasy Plus Micro extraction kit while all other samples were processed by Trizol Reagent extraction.

Extraction of total RNA from pituitary

Pituitary tissue was extracted using Qiagen RNeasy Micro kit including on-column DNase digestion according to the manufacturer´s protocol. All working steps were conducted at room temperature. Briefly, 2-mercaptoethanol was added to the lysis buffer (RLT buffer) before starting. Then, tissue was homogenized in 350 µL RLT buffer using an automatic bead mill (Tissue Lyser, Qiagen) and stainless steel beads (Ø 5 mm, Qiagen; 2 x 2 min, 18 s^{-1}). After 3 min of centrifugation (12,000 rpm; Biofuge Fresco, Heraeus, Hanau), supernatant was transferred to gDNA eliminator column and centrifuged again (15 s, 10,000 rpm). Supernatant was transferred into collection tube filled with 350 µL EtOH (70 %) and mixed to achieve RNA precipitation. The mixture was immediately transferred to RNeasy MinElute spin columns and centrifuged (15 s, 10,000 rpm). The eluate was discarded and the spin columns were washed with 700 µL RW1 buffer. Again, the eluate was discarded and the washing step was repeated with 500 µL RW1 buffer. After washing with 500 µL RPE buffer, columns were centrifuged (15 s, 10,000 rpm) and 500 µL EtOH (80 %) were added. Then, samples were centrifuged again (2 min, 10,000 rpm), the eluate was discarded and centrifugation was done again with open column lids to completely eliminate the EtOH. Finally RNA was eluted from the silica membrane of the spin columns using 14 µL nuclease free water. Samples were stored at -80 °C until further processing.

Extraction of total RNA from primary hepatocytes or tissue samples (other than pituitary)

Total RNA was obtained by using a phenol-chloroform extraction with Trizol Reagent according to the manufacturer´s protocol with little modifications. Frozen samples were homogenized in 700 µL Trizol Reagent using an automatic bead mill (Tissue Lyser, Qiagen) and stainless steel beads (Ø 5 mm, Qiagen). The Tissue Lyser was set on a shaking frequencies of 18 s^{-1} and homogenization was achieved by shaking two times for 1 min 30 s. Then, 600 µL of Trizol Reagent were added. Samples were centrifuged (Biofuge Fresco, Heraeus) for 10 min at 12,000 rpm (4 °C) and 1000 µL of the clear Trizol-phase were transferred into a new reaction tube. Depending on the color of the homogenate up to 300 µL

Trizol Reagent were added. Chloroform was added depending on the amount of Trizol Reagent (250 µL Chloroform upon 1300 µL Trizol Reagent). Samples were mixed vigorously (15 s) and incubated for 10 min at room temperature afterwards. Phases were separated by centrifugation (15 min, 12,000 rpm, room temperature) and 300 µl of the upper, aqueous phase were transferred into new reaction tubes and mixed with the equal volume of isopropanol. For RNA precipitation, samples were incubated at room temperature for 10 min and then another 60 min at -20 °C. After centrifugation for 12 min at 12,000 rpm (4 °C), the supernatant above the obtained RNA pellet was removed and the pellet was washed with 300 µl ice-cold EtOH (70 %). Again, samples were centrifuged (6 min, 12,000 rpm, 4 °C), supernatant was removed and pellets were dried at room temperature. Dry pellets were cooled on ice and dissolved in nuclease free water depending on the size of the pellet (20-30 µL). Samples were then processed for RNA concentration measurements or stored at -80 °C until further analyses.

2.4.6.2 Determination of total RNA concentration

The amount and purity of extracted RNA was quantified by UV absorbance measurements using a Nanodrop ND-1000 spectrophotometer (NanoDrop Products, Thermo Fisher Scientific, Schwerte, Germany). The RNA concentration is based on the absorbance measurements at 260 nm (ng μL^{-1}), while the purity of RNA is based on the ratios of absorbance measurements at 260 nm and 280 nm as well as at 230 nm and 280 nm. Samples of the same extraction series were diluted to the smallest possible RNA concentration, ideally set to 0.125 ng μL^{-1}.

2.4.6.3 Determination of RNA integrity

The determination of the RNA quality based on the RNA integrity number (RIN) was performed for a randomly chosen subset of samples from each extraction using Agilent RNA 6000 Nano kits analyzed in an Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). The RNA integrity is crucial for applications such as quantitative real time PCR (qPCR; Fleige and Pfaffl 2006). Size separation of the RNA molecules, especially ribosomal RNA 18S rRNA and 28S rRNA is necessary and automatically analyzed by chip-based micro capillary gel electrophoresis. An internal RNA marker and RNA ladder are sued as standards during the electrophoresis to allow molecule size estimations of RNA species. Prior to loading the chips, aliquots of RNA samples were denatured for 2 min at 70 °C (Thermocycler, Biometra). Chips were prepared according to the manufacturer´s protocol and 1 µL of each

sample (RNA concentration between 25-500 ng μL^{-1}) was analyzed (eukaryotic total RNA setting). Corresponding RIN scores were calculated by the 2100 expert software (version B.02.05.SI360, Agilent Technologies). A RIN score of 10 indicates an intact RNA while a score of 1 characterizes a highly degraded RNA (Schroeder et al. 2006). The RIN scores of all samples analyzed were > 8 and therefore, RNA samples were of sufficient integrity.

2.4.6.4 DNase treatment

Prior to reverse transcription (RT), RNA from samples other than pituitary were treated with DNase I to remove contaminations of genomic DNA. A total of 1 µg RNA in 10 µL reaction volume was incubated for 15 min at room temperature with 1 µL reaction buffer and 1 µL DNase I. Immediately after incubation, DNase I was inactivated by adding 1 µL EDTA to the reaction mixture and incubation at 65 °C for 10 min was carried out in a thermal cycler (Biometra, Göttingen, Germany).

2.4.6.5 Complementary DNA synthesis by reverse transcription

All steps were performed on ice. Reverse transcription (RT) was carried out in a thermal cycler (Biometra, Göttingen, Germany). Due to a product replacement of the reverse transcriptase, different enzymes and protocols were applied for the *in vitro* and *in vivo* samples.

Reverse transcription of total RNA from in vitro experiments

Complementary DNA (cDNA) was reversely transcribed using Avian Myeloblastosis Virus Reverse Transcriptase (AMV-RT). For primer annealing, 17 µL of RNA (55 ng μL^{-1}) was heated together with 3 µL poly(dt)-primer (1:40 diluted), having a final concentration of 4 µM, at 70 °C for 3 min, and placed on ice afterwards. To perform reverse transcription 10 µL premix containing 5 µL nuclease free water, 3 µL AMV-RT buffer, 1.5 µL dNTPs and 0.5 µL AMV-RT were added to each sample. To test for the potential presence of genomic contaminations, negative control samples were prepared (0.5 µL nuclease free water instead of AMV-RT). Incubation was carried out at 37 °C for 60 min and terminated by heating up to 94 °C for 2 min. Samples were stored at −20 °C until used for qPCR.

Reverse transcription of total RNA from in vivo experiments

cDNA was reversely transcribed using Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV-RT). For primer annealing, 6 µL of RNA (55 ng μL^{-1}) was heated

together with 4 µL of premix 1 (3 µL nuclease free water + 1 µL poly(dt)-primer, 1:10 diluted) at 65 °C for 2 min, and placed on ice for 1 min afterwards. To perform reverse transcription 10 µL premix 2 containing 4.5 µL nuclease free water, 2 µL buffer, 2 µL DTT, 1 µL dNTPs and 0.5 µL MMLV-RT were added to each sample. To test for the potential presence of genomic contaminations, negative control samples were prepared (0.5 µL nuclease free water instead of MMLV-RT). Incubation was carried out at 37 °C for 60 min and terminated by heating up to 85 °C for 5 min. Samples were stored at -20 °C until used for qPCR.

2.4.6.6 Gene expression analyses by quantitative real time PCR

All qPCR reactions were run in a Stratagene MX3000 or 3005 real-time PCR cycler (Agilent, Böblingen, Germany) using Platinum Taq DNA polymerase. PCR reactions for all gene expression analyses were run with 2 µL cDNA in 20 µL reaction volume (0.4 µM primer, 1x PCR buffer, 2-3.0 mM $MgCl_2$, 0.5 mM dNTPs, 0.1x SYBR-Green solution and 1 U Platinum Taq Polymerase) under the following thermal cycling conditions: initial denaturation at 95 °C for 7 min 40 s, followed by 40 cycles of denaturation at 95 °C for 17 s, primer annealing for 25 s (see Table 7 for annealing temperatures) and extension at 72 °C for 25 s. Reference samples (calibrator) used to determine relative amounts of target transcript were produced by pooling cDNA samples from different treatments. PCR reactions were run in duplicates for all samples. qPCR data were analyzed using MxPro software (Stratagene) by means of the comparative C_T method ($\Delta\Delta C_T$), including the corresponding amplification efficiencies (Pfaffl 2001). The housekeeping gene elongation factor 1α (EF1-a) was used as an internal standard by performing each PCR of any target gene and EF1-a from the same samples. Expressions of target genes were normalized to the corresponding level of EF1-a mRNA. In addition to samples without reverse transcriptase, no template control reactions (NTC, cDNA was replaced by nuclease free water) served as additional negative controls to check for the specificity of target cDNA amplification.

2.4.6.7 Primer design and efficiency calculations

Transcript-specific *O. niloticus* primers (Table 7) were either taken from literature sources (GST: Costa et al. 2012; MDRP: Ziková et al. 2010a, GH and IGF-I: Ziková et al. 2010b, LH and FSH: Mahdi El-Nadi 2011) or newly designed according to sequences published in the National Center for Biotechnology Information (NCBI), using Primer 3 (http://primer3.wi.mit.edu/). All primers were synthesized by TibMol (Berlin, Germany) and

cycle numbers, annealing temperatures, $MgCl_2$ and primer concentrations were optimized in a temperature gradient thermocycler (Eppendorf). PCR products were sized by gel electrophoresis (Agagel electrophoresis unit, Biometra) in a 2.0 % agarose gel in TAE-buffer (70 mV for 60 min). Therefore, 4 µL of PCR solution were mixed with 1 µL loading dye and transferred into the agarose gel. On each gel a 50 bp DNA-ladder (0.1 mg/mL) was included as a molecular size marker. Gels were analyzed using the gel documentation system GelDoc 2000 (Biorad). Bands of PCR products of the expected size were purified from the agarose gels using QIAquick gel extraction kit (Qiagen). DNA concentrations of PCR samples were determined spectrophotometrically using the NanoDrop device and loading capacity of the columns was not exceeded. Five volumes of PB buffer were added to each PCR sample and the mixture was transferred to a QIAquick spin column, centrifuged for 30 s at 13,000 rpm and the flow-through was discarded. The columns were washed with 750 µL PE buffer, followed by centrifugation (30 s, 13,000 rpm). After drying the columns by centrifugation (1 min, 13,000 rpm), the DNA was eluted with 50 µL PCR water. Purified PCR products were stored at -20 °C. Sequencing of purified PCR products was performed by Sequence Laboratories (Göttingen, Germany). Specific amplification was confirmed by the data base homology search tool (BLAST). For each set of primers, the efficiency of the PCR reaction was determined by analyzing five-fold serial dilutions (spanning at least four orders of magnitude) of pooled cDNA in duplicates. Standard curves were calculated by blotting the cycle threshold (C_T) against the logarithm of the respective cDNA dilution (Pfaffl 2001). Resulting slopes from regression fit equations were used to determine the amplification efficiency according to: Efficiency = $10^{(-1/slope)}$. Amplification efficiencies ranged between 85 and 114 %.

Table 7. Primer used for gene expression analysis by real time PCR. bp: base pairs; Ta: annealing temperature.

Target gene	Forward (f) and reverse (r) primer 5'-3' sequence	Product length [bp]	Ta [°C]	NCBI accession number
elongation factor 1α (EF-1a)	f: gCTTCAACgCTCAggTCATC r: TgTgggCAgTgTggCAATC	86	62	AB075952.1
cytochrome P4501A monooxygenase (CYP1A)	f:CTgACCTgTACAgCTTTCgC r: gCACATgAgTACTCTggggT	153	62	AB048938.1
glutathione- S-transferase (GST)	f: AAATggATggCATgAAgCTC r: TCgTTCTTTgggATCCTTTg	92	60	DQ397879
multidrug resistance protein (MDRP)	f: CAgATgggAATAAAgAAggCgA r: TgTCgATACTggggACgTgAT	273	58	EU878755.1
vitellogenin (VTG)	f: CTTTCCATCCAgCCACCAAg r: CTgCAggAggTTgATgATgC	160	60	FJ709597.1
growth hormone (GH)	f: CTgCTgATCAgggCCAATC r: TCgACATTTAgCTACCgTCAgg	191	58	M97765.1 M97766.1
insulin-like growth factor I (IGF-I)	f: AgTTTgTCTgTggAgAgCgAg r: gTgTgCCgCTgTgAACg	188	58	EU272149.1
follicle stimulating hormone (FSH)	f: TgTCgCCCAAAgAACATCAgC r: TTCACCTCgTAggACCACTCTC	158	62	AY294015.1
luteinizing hormone (LH)	f: ACCAAggACCCTgTCATC r: gCAgTggCAACTCAAAgC	152	62	AY294016.1

2.4.7 Histology

The third left gill arches of eight experimental fish from the long-term exposure experiments were evaluated histologically after 80 dph based on microscopic analyses of histopathological alterations. All chemicals and reagents used are listed in Table 8.

Table 8. Chemical and reagents used for histological processing.

Reagents	Description	Supplier
ethanol (EtOH)	≥ 99.8 %, denatured; diluted with aqua dest.	Roth, Karlsruhe, Germany
xylene	98 %; for histology	Roth, Karlsruhe, Germany
paraffin	Paraplast PLUS, tissue embedding medium	Roth, Karlsruhe, Germany
eosin	concentrate for microscopy; diluted to 0.1 % with aqua dest.	Roth, Karlsruhe, Germany
hematoxylin	according to Harris	Roth, Karlsruhe, Germany
DePex	mounting medium	VWR

2.4.7.1 Dehydration and paraffin embedding

Gill tissue samples were dehydrated using an automatic tissue processor (Shandon Excelsior^{TM} ES, Thermo Scientific) according to the protocol given in Table 9. Samples were rinsed in a graded series of ethanol, xylene and paraffin.

Table 9. Protocol for tissue dehydration and paraffin embedding.

Step	Solution	Temperature	Time (h:min)
1.	75 % EtOH	room temperature	1:00
2.	90 % EtOH	room temperature	1:00
3.	95 % EtOH	room temperature	1:00
4.	95 % EtOH	room temperature	1:00
5.	100 % EtOH	room temperature	1:00
6.	100 % EtOH	room temperature	1:00
7.	xylene	room temperature	1:00
8.	xylene	room temperature	1:00
9.	xylene	room temperature	1:00
10.	paraffin	60 °C	1:20
11.	paraffin	60 °C	1:20
12.	paraffin	60 °C	1:20

Using an embedding centre consisting of a paraffin-dispenser (PA/5.9; C+E), a preheating unit (PA/W; C+E) and a paraffin stretching table (DDM-P064; MDS Group, Buseck, Germany) samples were placed into embedding moulds containing freshly melted paraffin. Gill arches were directed in a way that the primary filaments pointed to the right side.

2.4.7.2 Sectioning and staining

Samples were cut into 4 µm slices using a rotary microtome (Jung Supercut 2065; Leica, Wetzlar, Germany). Serial sections were transferred to a water bath (40 °C) for stretching out wrinkles, then mounted on microscope slides (Superfrost; Roth, Karlsruhe, Germany) and heat fixed at 60 °C overnight. Samples were stored at room temperature until staining. Staining was performed with hematoxylin and eosin according to Table 10.

Table 10. Protocol for hematoxylin-eosin staining.

Step	Solution	Time (min:s)
1.	xylene	5:00
2.	xylene	5:00
3.	100 % EtOH	2:00
4.	96 % EtOH	2:00
5.	70 % EtOH	2:00
6.	40 % EtOH	2:00
7.	aqua dest	2:00
8.	hematoxylin	2:00
9.	running tap water	10:00
10.	eosin (0.1 %)	1:00
11.	aqua dest.	0:15
12.	80 % EtOH	2:00
13.	96 % EtOH	2:00
14.	100 % EtOH	2:00
15.	100 % EtOH	2:00
16.	xylene	5:00
17.	xylene	5:00

2.4.7.3 Microscopic examination

Histological evaluation was performed with gill samples from eight fish per treatment group. Samples were analyzed blindly at 1000 x magnification using a Olympus BX50 microscope equipped with a XC50 digital camera (Olympus, Hamburg, Germany). A total of 150 secondary lamellae (SLs) per fish were analyzed. Therefore, five primary filaments per sample and 30 SLs per primary filament were chosen. Counting was started in the middle of the primary filaments. Dorsal and ventral secondary lamellae were included in same amounts. After a general survey of occurring pathological alterations according to Mallat (1985), six categories were defined for analysis: (a) not affected, (b) epithelial lifting, (c) hyperplasia (including lamellar fusion), (d) proliferation of mucous cells, (e) hypertrophy of chloride cells and (f) infiltration by leucocytes. Within these categories four degrees of severity were established depending on the area impaired or the amount of cells involved, respectively. Ranking was: without lesion = (-) , mild = (+), moderate = (++) and severe = (+++).

2.4.8 Oxidative stress evaluation

LPO is an often used indices for oxidative stress. The level of LPO was analyzed by the thiobarbituric acid reactive substances (TBARS) assay according to Lushchak et al. (2005) with some modifications. The decomposition of lipid hydroperoxides produces low molecular weight products, including malondialdehyde, which can be measured by the TBARS assay. Frozen tissue samples were homogenized (1:10 w/v) in 20 mM sodium phosphate (NaP) buffer, pH 7.0, containing 0.5 mM EDTA using an Ultra Turrax (Ika, Staufen, Germany). An aliquot of the homogenates (ideally 250 µL) was mixed with the same volume of sodium dodecyl sulfate (SDS; 7 %) and incubated for 5 min at room temperature. After incubation, samples were immersed in ice cold water and twice the volume of the initial aliquot of a solution containing 0.8 M HCl and 12.5 % trichloroacetic acid (TCA) was added. The same volume of thiobarbituric acid (TBA; 1 %, freshly prepared) was added. Samples were incubated for 45 min at 95 °C in a water bath. After quick cooling in ice cold water, the same butanol volume as the total mixture volume was added and samples were mixed vigorously. Samples were centrifuged for 10 min, 4 °C at 4,700 rpm and the butanol phase was removed and used for absorption measurements at 535 nm (Infinite 200, Tecan, Männedorf, Switzerland). Concentrations were calculated using a standard curve based on different concentrations of malondialdehyde (1,1,3,3-tetraethoxipropane) and are expressed in nmol of TBARS per gram wet weight of tissue.

2.4.9 Data analyses

Data is presented as mean ± standard deviation of the mean (SD) or indicated otherwise. All statistical analyses were done using Graphpad Prism 4 software (GraphPad Software, Inc.). Data was tested for criteria of normality (Kolmogorov-Smirnov test) and homogeneity of variance (Barlett´s test) and transformed if necessary.

Data obtained from the *in vitro* experiments was analyzed by paired t-test (parametric data) or Wilcoxon test for paired values (non-parametric data). Due to limited numbers of cell cultures (five fish with at least two technical replicates per fish and treatment) the significance level was set to $p \leq 0.06$.

Beside the statistical evaluation of the impaired secondary lamellae (SLs) based on the severity levels, data obtained from the *in vivo* experiments was analyzed by one-way analysis of variance (ANOVA) followed by Tukey´s HSD post hoc testing in case data met assumptions of parametric tests. In case data did not meet assumptions of normality and homogeneity of variance and transformation was not successful, Kruskal-Wallis one-way ANOVA was performed followed by Dunn´s multiple comparison test. Statistically significant differences between the impaired SLs were analyzed using two-way ANOVA with number of SLs and severity level as two factors, followed by Bonferroni post testing. In all cases, $p < 0.05$ was considered to be statistically significant.

3 Results

3.1 *In vitro* exposure to DCF and MTP

During the experiment, *O. niloticus* primary hepatocytes could be kept alive. The integrity of the cells was verified using a microscope as described by Segner et al. (1993). According to a positive control, the expression level of a housekeeping gene (EF1-a) was nearly constant in all samples (data not shown).

3.1.1 Cytochrome P4501A monooxygenase

After 24 h exposure to DCF, biotransformation phase I enzyme CYP1A was statistically significant up-regulated in primary hepatocytes of *O. niloticus* for all three test concentrations compared to control treatment (Figure 12). The two lower test concentrations ($4x10^{-9}$ M DCF and $4x10^{-8}$ M DCF) showed an increase of about 1.2-fold, while the highest test concentration ($4x10^{-7}$ M DCF) led to an 1.6-fold increase. Exposure to MTP induced CYP1A gene expression of about 1.2 to 1.3-fold for all three concentrations tested. However, the change in CYP1A mRNA expression was only statistically significant at $4x10^{-7}$ M DCF compared to the control.

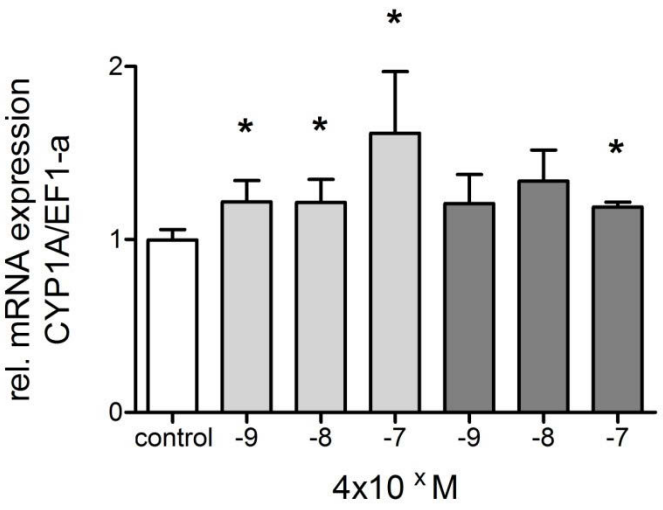


Figure 12. Relative mRNA expression of cytochrome P4501A monooxygenase (CYP1A) in primary hepatocytes of *Oreochromis niloticus* after 24 h exposure to 0 (control), $4x10^{-9}$ M, $4x10^{-8}$ M and $4x10^{-7}$ M diclofenac (light gray) and metoprolol (dark grey), respectively. Results (mean and SD) are expressed relative to control values and normalized to elongation factor 1-α (EF1-a) (n=5 with at least two technical replicates per fish and treatment). Asterisks indicate statistically significant differences to control (t-test for paired values, *$p \leq 0.06$).

3.1.2 Glutathione-S-transferase

Relative mRNA expression of GST increased dose-dependently for all DCF concentrations (Figure 13). While the two lower test concentrations ($4x10^{-9}$ M and $4x10^{-8}$ M DCF) caused an increase of about 2-fold, the increase due to the highest test concentration ($4x10^{-9}$ M DCF) was about 4.3-fold. Exposure to MTP changed GST expression levels statistically significant only at the lowest concentration of $4x10^{-9}$ M MTP (1.8-fold) compared to control treatment. The two higher concentrations increased GST gene expression between 2.2 and 1.6-fold.

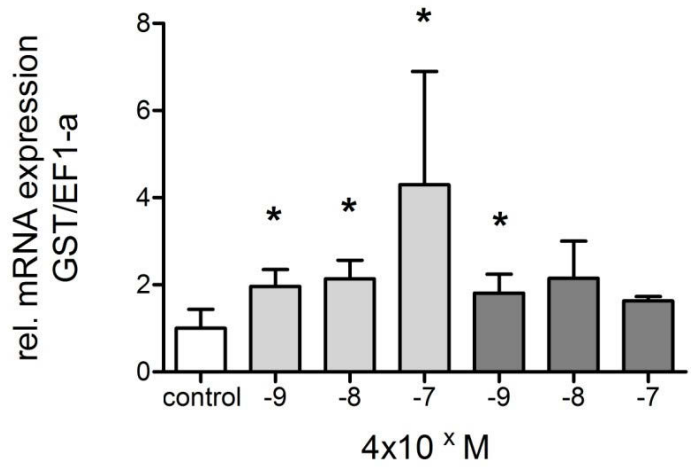


Figure 13. Relative mRNA expression of glutathione-S-transferase (GST) in primary hepatocytes of *Oreochromis niloticus* after 24 h exposure to 0 (control), $4x10^{-9}$ M, $4x10^{-8}$ M and $4x10^{-7}$ M diclofenac (light gray) and metoprolol (dark grey), respectively. Results (mean and SD) are expressed relative to control values and normalized to elongation factor 1-α (EF1-a) (n=5 with at least two technical replicates per fish and treatment). Asterisks indicate statistically significant differences to control (Wilcoxon test for paired values, *$p \leq 0.06$).

3.1.3 Multidrug resistance protein

Relative mRNA expression levels of MDRP were statistically significant different compared to the control after 24 h exposure to $4x10^{-9}$ M DCF (Figure 14). Expression levels tended to increase with elevating concentrations (between 1.9 to 3.0-fold). MTP did not significantly change MDRP mRNA levels at any concentration tested, but mean values were between 1.8 and 2.7-fold higher compared to the control treatment.

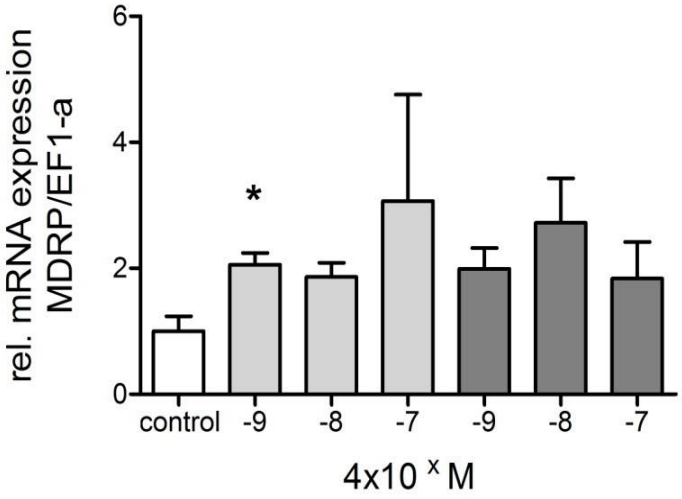


Figure 14. Relative mRNA expression of multidrug resistance protein (MDRP) in primary hepatocytes of *Oreochromis niloticus* after 24 h exposure to 0 (control), $4x10^{-9}$ M, $4x10^{-8}$ M and $4x10^{-7}$ M diclofenac (light gray) and metoprolol (dark grey), respectively. Results (mean and SD) are expressed relative to control values and normalized to elongation factor 1-α (EF1-a) (n=5 with at least two technical replicates per fish and treatment). Asterisks indicate statistically significant differences to control (Wilcoxon test for paired values,*$p \leq 0.06$).

3.1.4 Vitellogenin

With increasing concentrations of DCF, relative mRNA expression of VTG in *O. niloticus* primary hepatocytes increased (1.3 to 3.0-fold; Figure 15). In comparison to the control group, this increase was statistically significant for $4x10^{-9}$ M and $4x10^{-7}$ M DCF. Exposure to MTP increased VTG mRNA expression statistically significant only at the second highest test concentration ($4x10^{-8}$ M MTP; 1.6-fold) compared to the control group. EE2 induced VTG mRNA expression statistically significant compared to the control group (2.0-fold).

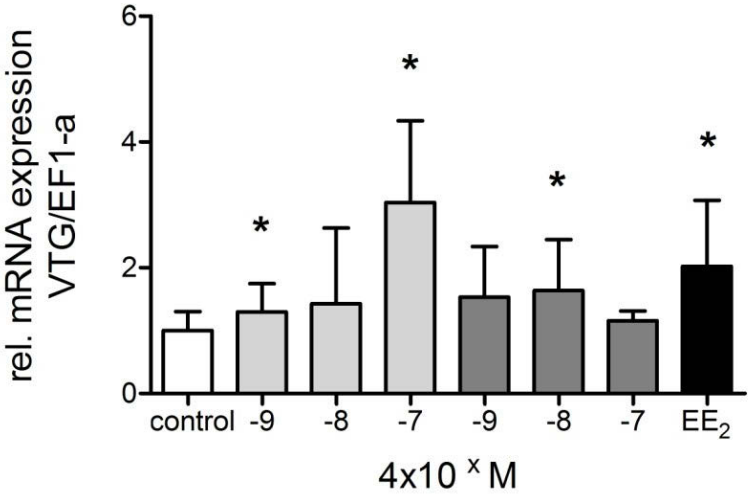


Figure 15. Relative mRNA expression of vitellogenin (VTG) in primary hepatocytes of *Oreochromis niloticus* after 24 h exposure to 0 (control), $4x10^{-9}$ M, $4x10^{-8}$ M and $4x10^{-7}$ M diclofenac (light gray) and metoprolol (dark grey), respectively. EE_2 (17 α-Ethinylestradiol; 10^{-6} M) was used as positive control. Results (mean and SD) are expressed relative to mean control and normalized to elongation factor 1-α (EF1-a) (n=5 with at least two technical replicates per fish and treatment). Asterisks indicate statistically significant differences to control (Wilcoxon test for paired values, *$p \leq 0.06$).

3.2 *In vivo* exposure

Water parameters could be kept nearly constant throughout the *in vivo* experiments (Table 11), confirming optimal experimental conditions. In the following, nominal concentrations are used instead of the effective ones to simplify the denotation of the different treatment groups.

Table 11. Water parameters throughout the long-term exposure experiments.

Substance	Water temperature [°C]	pH	Oxygen [mg/L]
diclofenac	27.4 ± 0.4	7.62 ± 0.30	7.11 ± 0.55
metoprolol	27.4 ± 0.4	7.66 ± 0.25	7.16 ± 0.42

3.2.1 Diclofenac

3.2.1.1 DCF analytics

DCF was not detectable in the control treatment. The effective DCF concentrations in the test water samples of the treatment concentrations were in the range of 99-112 % of the nominal values during the exposure experiment (Table 12). The Organization for Economic Co-operation and Development (OECD) test guidelines require 80 to 120 % of nominal and therefore, measured values are considered to be reliable.

Table 12. Diclofenac exposure experiment. Nominal concentrations and mean ± standard deviation (SD) of the actual exposure concentrations [µg L^{-1}] during the entire experiment, including percentage of the nominal concentrations. n.d.: not detectable.

Treatment [M]	**Nominal concentration [µg L^{-1}]**	**Mean measured concentration ± SD [µg L^{-1}]**	**% of nominal concentration ± SD**
control	0	n.d.	n.d
$4x10^{-10}$	0.13	0.15 ± 0.03	112 ± 20
$4x10^{-9}$	1.27	1.37 ± 0.17	107 ± 14
$4x10^{-8}$	12.72	12.59 ± 2.76	97 ± 23
$4x10^{-7}$	127.24	137.71 ± 30.59	111 ± 25

3.2.1.2 Hatching

Hatching of *O. niloticus* larvae was completed after four days of exposure. The mean hatching rate of the control group was 99 ± 2 % (mean ± SD). Hatching success was not significantly affected by DCF exposure. For all treatment groups mean hatching success was almost equal to the control group, ranging from 96 ± 5 % to 99 ± 3 % (mean ± SD) (Figure 16).

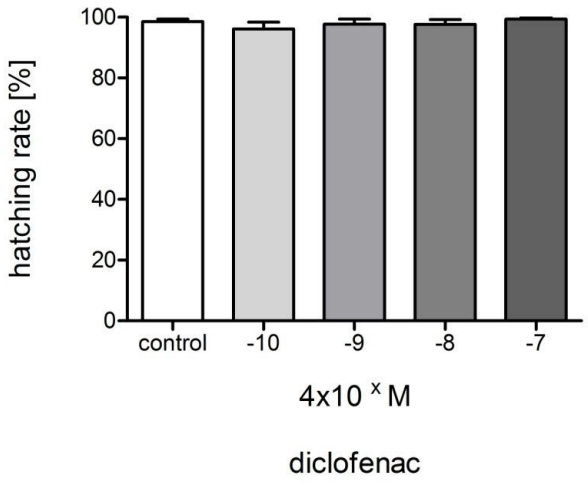


Figure 16. Hatching rates [%] of *Oreochromis niloticus* exposed to diclofenac (mean and SD). Data was analyzed using Kruskal-Wallis test revealing no statistical significant differences between treatment groups.

3.2.1.3 Survival

Survival of *O. niloticus* was not affected by DCF exposure (Figure 17). Mean survival of the control fish throughout the experiment was 69 ± 9 % (mean ± SD), while the treatment groups ranged between 65 ± 15 % to 80 ± 12 % (mean ± SD) and did not differ significantly from the control. Almost no fish died until 8 dph but for all treatment groups, mortality was found to be highest between 9 to 30 dph of exposure (Figure 18).

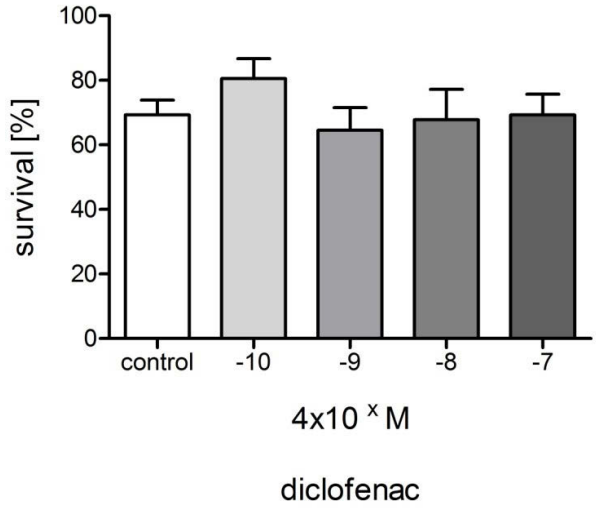


Figure 17. Survival [%] of *Oreochromis niloticus* exposed to diclofenac for 80 days post-hatch (mean and SD). Data was analyzed using Kruskal-Wallis test revealing no statistical significant differences between treatment groups.

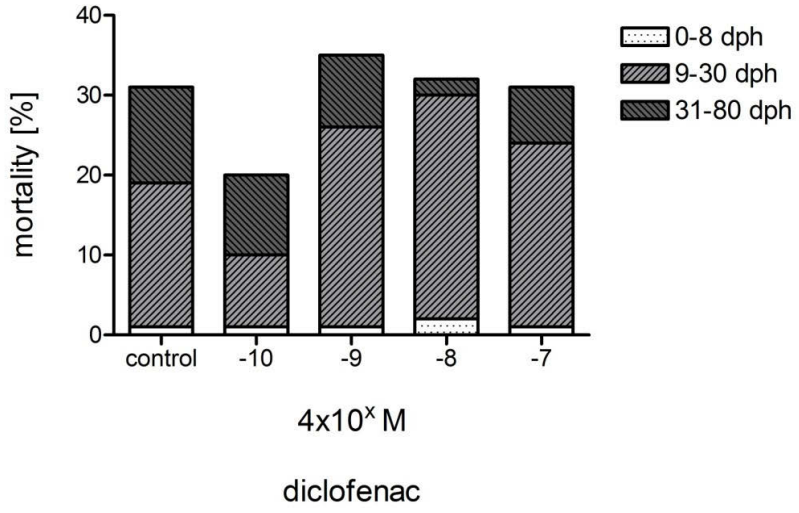


Figure 18. Cumulative mortality [%] of *Oreochromis niloticus* exposed to diclofenac for various time phases until 80 days post-hatch (dph).

3.2.1.4 Growth

After 8 and 30 dph of DCF exposure, fish wet weight was not impaired by any DCF treatment concentration (Figure 19 A/B). After 80 dph wet weight of exposed fish was slightly reduced and ranged between 92 - 97 % of the control group (Figure 19 C). Exposure to $4x10^{-8}$ M DCF

reduced wet weight significantly compared to the control group. The same pattern was found for fish length (Figure 20).

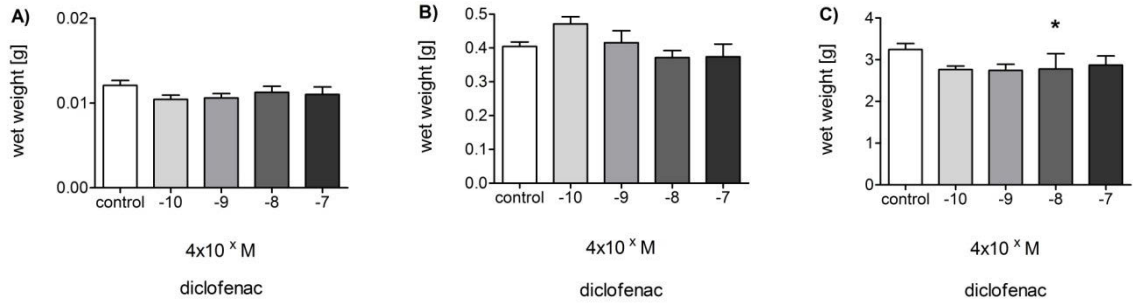


Figure 19. Wet weight [g] of *Oreochromis niloticus* after (A) 8, (B) 30 and (C) 80 days post-hatch of diclofenac exposure, respectively. The arithmetic means and SD were calculated from the mean values of the four tank replicates per treatment (n=11 or 12 at all sampling points). Asterisks indicate statistically significant differences to control (Dunn´s Multiple Comparison test, *p < 0.05).

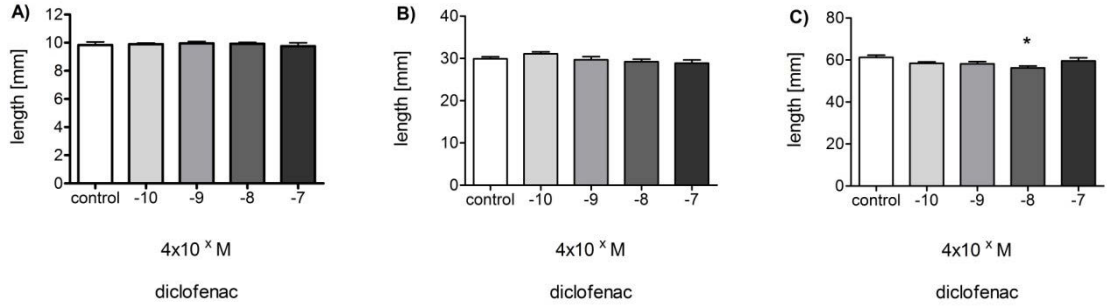


Figure 20. Length [mm] of *Oreochromis niloticus* after (A) 8, (B) 30 and (C) 80 days post-hatch of diclofenac exposure, respectively. The arithmetic means and SD were calculated from the mean values of the four tank replicates per treatment (n=11 or 12 at all sampling points). Asterisks indicate statistically significant differences to control (Tukey´s Multiple Comparison test, *p < 0.05).

Based on the individual length and wet weight, the condition index (CI) was calculated (Table 13). After 8 dph, CI did not differ statistically significant, although the CI of the control group was slightly higher than in the DCF exposure groups. Only after 30 dph, $4x10^{-8}$ M DCF reduced CI statistically significant compared to the control group. Again, no significant changes were found after 80 dph.

Table 13. Condition index [g/cm³] of *Oreochromis niloticus* exposed to diclofenac for 8, 30 and 80 days post-hatch (dph), respectively. Means and standard deviation (SD) are presented (n=11 or 12 at all sampling points). Asterisks indicate statistically significant differences to control (Dunn´s Multiple Comparison test, *p < 0.05).

	8 dph	**30 dph**	**80 dph**
control	1.31 ± 0.37	1.53 ± 0.25	1.40 ± 0.05
4x10^{-10} M	1.08 ± 0.23	1.56 ± 0.08	1.38 ± 0.06
4x10^{-9} M	1.07 ± 0.18	1.55 ± 0.07	1.38 ± 0.06
4x10^{-8} M	1.15 ± 0.22	1.47 ± 0.06 *	1.56 ± 0.71
4x10^{-7} M	1.19 ± 0.31	1.50 ± 0.09	1.33 ± 0.07

Effects of DCF exposure on the growth axis were partly analyzed, namely by gene expression analysis of growth hormone (GH, Figure 21 A) and insulin-like growth factor I (IGF-I, Figure 21 B). No statistically significant changes in gene expression were found, but GH showed the tendency to increase while there was a constant decrease in IGF-I mRNA expression with increasing DCF concentrations. Exposure to 4x10^{-7} M DCF caused a lower expression level of IGF-I of about 50 % compared to the control group.

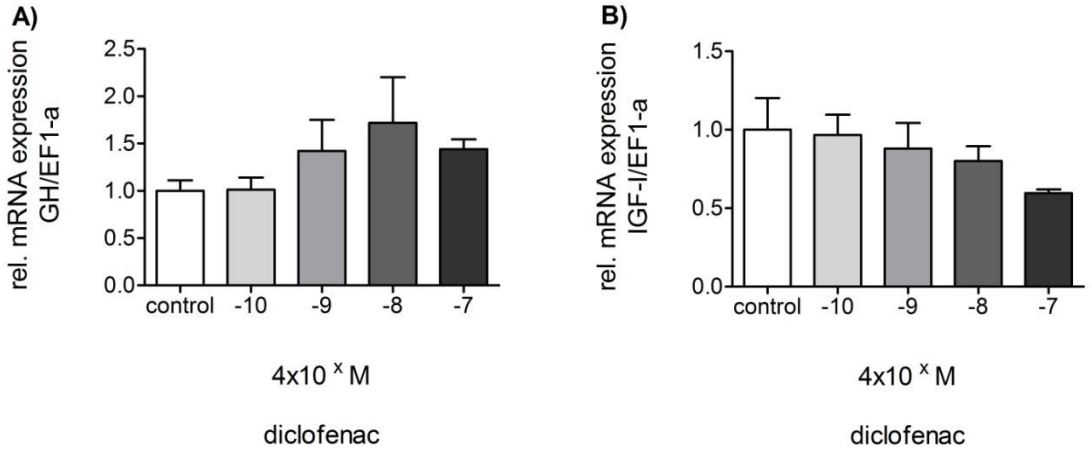


Figure 21. Relative mRNA expression of (A) growth hormone (GH) and (B) insulin-like growth factor I (IGF-I) in pituitary and liver, respectively, of *Oreochromis niloticus* after 80 days post-hatch of diclofenac exposure. Results (mean and SD) are expressed relative to control values and normalized to elongation factor 1-α (EF1-a) (n=8). Data was analyzed using Kruskal-Wallis test revealing no statistically significant differences between treatment groups.

3.2.1.5 Hepatosomatic index

The hepatosomatic index (HSI) increased statistically significant after 30 dph of DCF exposure (Table 14) with the highest value (3.33 ± 0.30 %) at the highest DCF treatment concentration. Compared to the control group, the increase was statistically significant for $4x10^{-9}$ M as well as $4x10^{-7}$ M DCF. Furthermore, $4x10^{-9}$ M had a greater HSI than $4x10^{-10}$ M. After 80 dph, the HSI was in general much smaller than after 30 dph, but no statistical differences between treatment groups occurred.

Table 14. Hepatosomatic index [%] of *Oreochromis niloticus* exposed to diclofenac for 30 and 80 days post-hatch (dph). Means and standard deviation (SD) are presented (n=11 or 12). Letters indicate statistically significant differences between treatments (Dunn´s Multiple Comparison test, $p < 0.05$).

	30 dph	**80 dph**
control	2.68 ± 0.79 a	0.60 ±0.07
$4x10^{-10}$ M	2.56 ± 0.43 ac	0.70 ± 0.14
$4x10^{-9}$ M	3.32 ± 0.11 b	0.70 ± 0.11
$4x10^{-8}$ M	3.05 ± 0.44 abc	0.64 ± 0.05
$4x10^{-7}$ M	3.33 ± 0.30 bc	0.59 ± 0.10

3.2.1.6 Histology

Using light microscopic examinations of gills of *O. niloticus* after 80 dph of exposure to DCF, various histopathological alterations could be observed. Overall, the DCF treatment groups showed statistically significant more SLs with histopathological alterations compared to the control group (except for $4x10^{-7}$ M DCF, Figure 22). All treatment groups showed around 70 % of SLs with alterations while in the control group less than 40 % were affected. In total, epithelial lifting and the proliferation of epithelial cells (hyperplasia) occurred most often, followed by hypertrophy of chloride cells (Figure 23). Within all categories, mild alterations were observed most often, but epithelial lifting also occurred notably in a moderate and severe manner. A statistically significant increase in certain alterations due to DCF exposure, however, was only determined for mild alterations of epithelial lifting (Figure 23 A and Figure 24 B), hypertrophy (Figure 23 B and Figure 24 D) and hyperplasia (Figure 23 C and Figure 24 C).

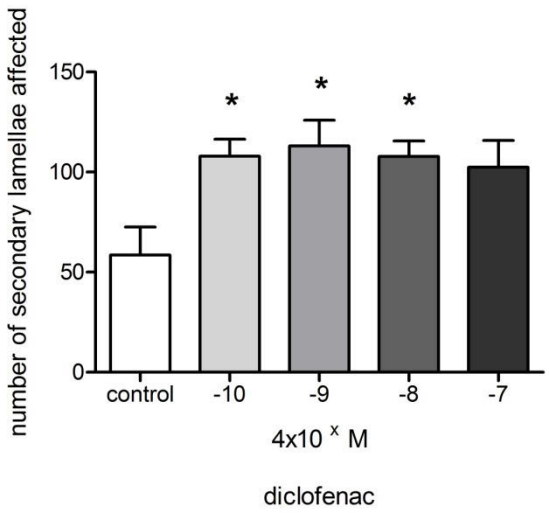


Figure 22. Total number of secondary lamellae (and SD) with histopathological alterations in gills of *Oreochromis niloticus* after 80 days post-hatch of diclofenac exposure (n=8). Asterisks indicate statistically significant differences to control (Tukey´s Multiple Comparison test, *$p < 0.05$).

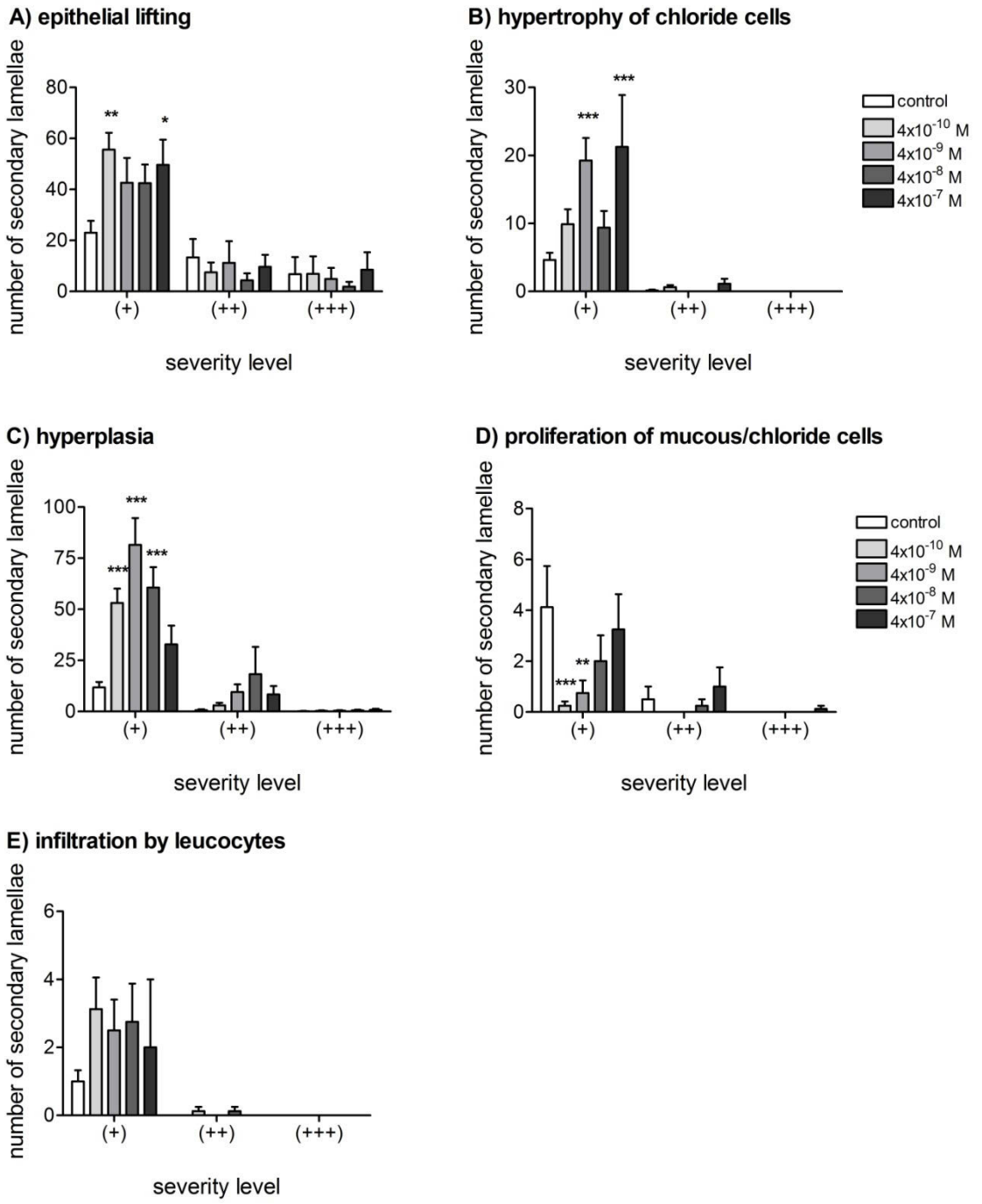


Figure 23. Mean number of secondary lamellae (out of 150) and SD in gills of *Oreochromis niloticus* with the following findings after 80 days post-hatch of diclofenac exposure: (A) epithelial lifting, (B) hypertrophy of chloride cells, (C) hyperplasia, (D) proliferation of mucous/chloride cells, (E) infiltration by leucocytes. Alterations are categorized: (+) mild, (++) moderate, (+++) severe. Note varying scales. Asterisks indicate statistically significant differences to control within one severity level of lamellar damage (Bonferroni post test, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

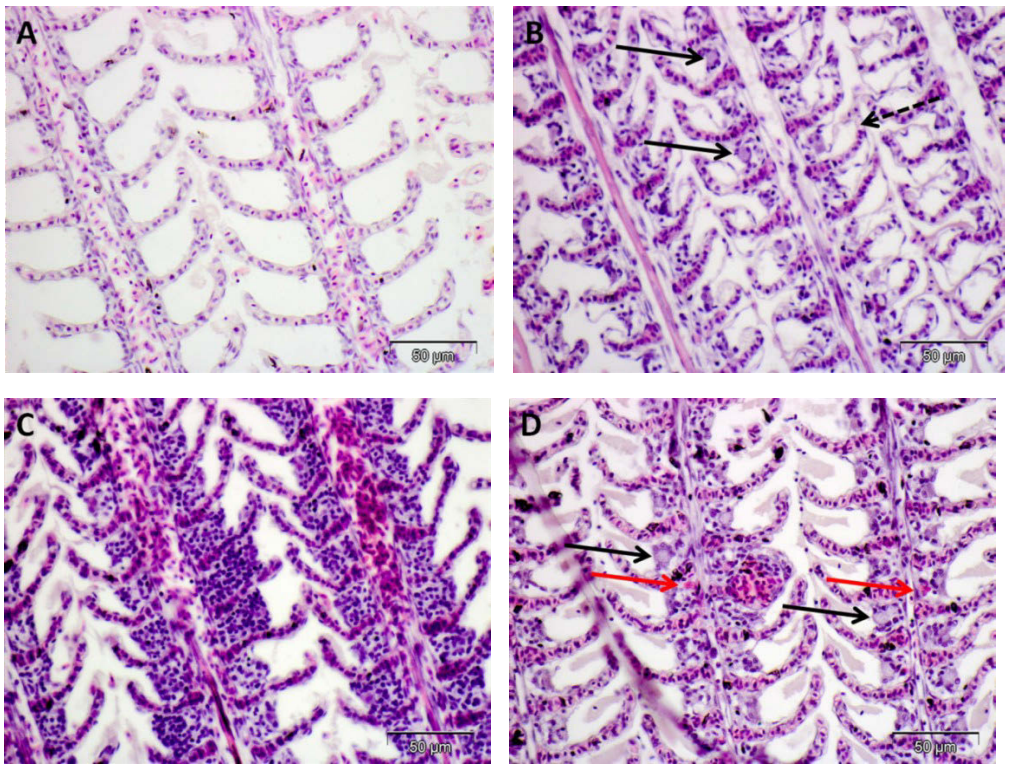


Figure 24. Gill histology of *Oreochromis niloticus* after 80 days post-hatch under experimental conditions. (A) control, (B-D) exposure to $4x10^{-7}$ M diclofenac. Histopathological alterations: (B) epithelial lifting (dotted black arrow) and hypertrophy of chloride cells (solid black arrows), (C) hyperplasia, (D) hypertrophy of chloride cells (solid black arrows), infiltration by leucocytes (eosinophile granulocytes, red arrows, occasionally observed). Original magnification 400 x, scale bar corresponds to 50 µm, sections of 4 µm thickness and stained by hematoxylin and eosin method.

3.2.1.7 Gene expression of pituitary gonadotropins and hepatic vitellogenin

Gene expression patterns of both pituitary gonadotropins, namely luteinizing hormone (LH) and follicle stimulating hormone (FSH) were analyzed to gain information about the influence of DCF exposure on the hypothalamus-pituitary-gonad axis (HPG axis, Figure 25). LH expression was statistically significant reduced with increasing DCF concentrations comparing the control group with $4x10^{-9}$ M and $4x10^{-7}$ M DCF (Figure 25 A). DCF exposure had no statistically significant effect on the expression levels of FSH (Figure 25 B).

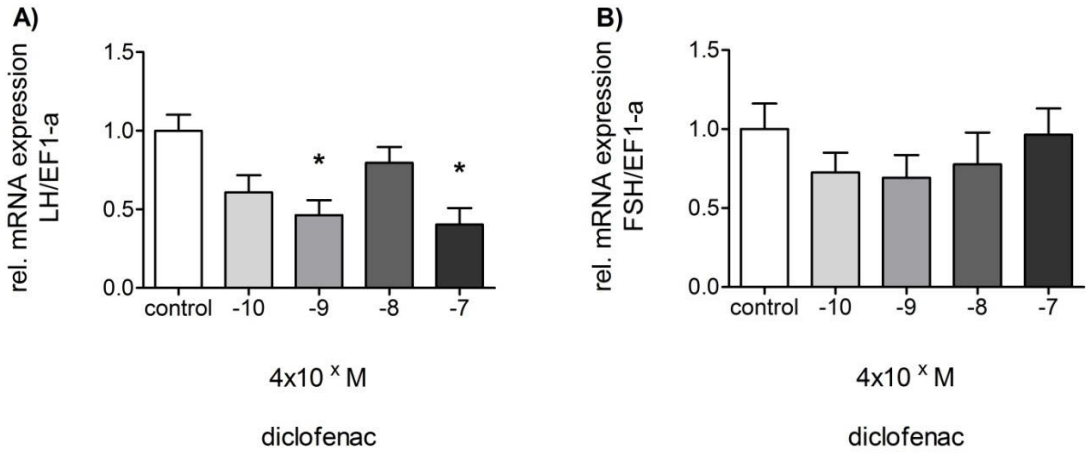


Figure 25. Relative mRNA expression of (A) luteinizing hormone (LH) and (B) follicle stimulating hormone (FSH) in the pituitary of *Oreochromis niloticus* after 80 days post-hatch of diclofenac exposure. Results (mean and SD) are expressed relative to control values and normalized to elongation factor 1-α (EF1-a) (n=6 to 8). Asterisks indicate statistically significant differences to control (Dunn´s Multiple Comparison test, *p < 0.05).

Relative mRNA expression of vitellogenin (VTG, Figure 26) increased in a non-monotonic dose response manner, with a distinct peak at $4x10^{-9}$ M DCF, leading to an inverted U-shape expression pattern. Overall, the increase was 1.5 to 3.8-fold. In comparison to the control group, this increase was statistically significant for $4x10^{-9}$ M DCF.

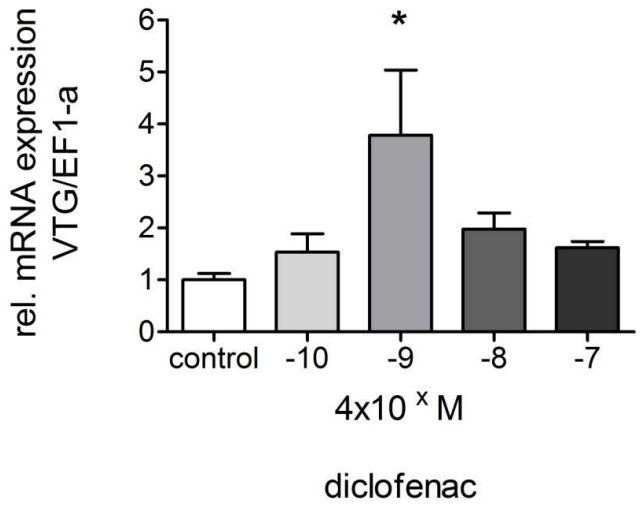


Figure 26. Relative mRNA expression of vitellogenin (VTG) in the liver of *Oreochromis niloticus* after 80 days post-hatch of diclofenac exposure. Results (mean and SD) are expressed relative to control values and normalized to elongation factor 1-α (EF1-a) (n=6). Asterisks indicate statistically significant differences to control (Tukey´s Multiple Comparison test, *p < 0.05).

3.2.1.8 Biotransformation

Relative mRNA expression of key enzymes of the three phases of the biotransformation pathways was analyzed at all sampling points, using whole larvae after 8 dph and liver samples after 30 and 80 dph, respectively. After 8 and 30 dph of DCF exposure no differences in expression patterns for all three enzymes were found (data not shown). After 80 dph (Figure 27), an induction was found with increasing DCF concentrations (with exception for $4x10^{-7}$ M DCF). The increase was statistically significant for GST at $4x10^{-9}$ M and $4x10^{-8}$ M DCF and for MDRP at $4x10^{-8}$ M DCF compared to the control group. MDRP induction was the highest with about 5-fold compared to the control group.

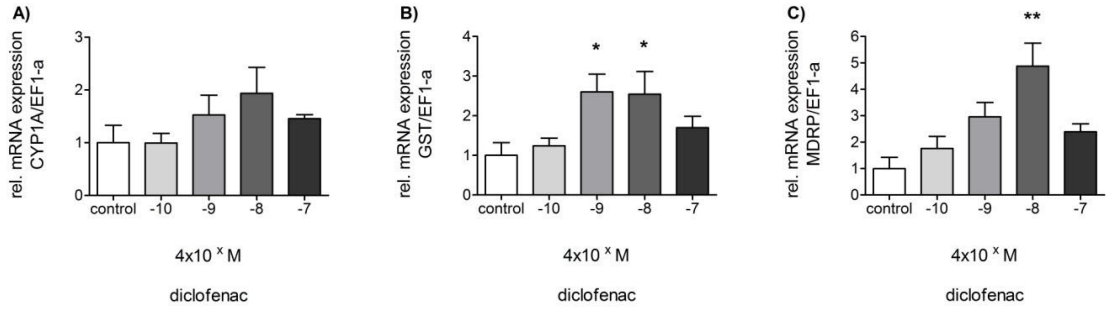


Figure 27. Relative mRNA expression of (A) cytochrome P4501A monooxygenase (CYP1A), (B) glutathione-S-transferase (GST) and (C) multidrug resistance protein (MDRP) in the liver of *Oreochromis niloticus* after 80 dph of diclofenac exposure. Results (mean and SD) are expressed relative to control values and normalized to elongation factor 1-α (EF1-a) (n=7). Asterisks indicate statistically significant differences to control (Dunn´s Multiple Comparison test *p<0.05, **p<0.01).

Additionally, CYP1A and GST gene expression was analyzed in gills of *O. niloticus* after 80 dph of DCF exposure (Figure 28). CYP1A gene expression was not statistically significant influenced by DCF exposure but showed the tendency to decrease with increasing DCF concentrations (Figure 28 A). GST gene expression was induced for the two lower DCF treatment groups and clearly reduced for the two higher DCF concentrations (Figure 28 B). The induction was statistically significant comparing the control group and $4x10^{-9}$ M DCF.

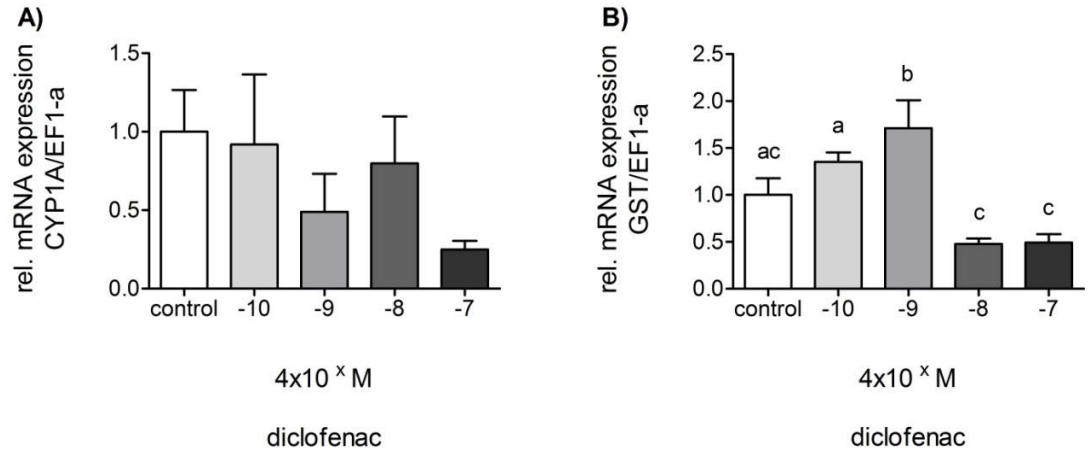


Figure 28. Relative mRNA expression of (A) cytochrome P4501A monooxygenase (CYP1A) and (B) glutathione-S-transferase (GST) in the gills of *Oreochromis niloticus* after 80 dph of diclofenac exposure. Results (mean and SD) are expressed relative to control values and normalized to elongation factor 1-α (EF1-a) (n=5 or 6). Letters indicate statistically significant differences between treatments (Tukey´s Multiple Comparison test, $p < 0.05$).

3.2.1.9 Oxidative stress

Overall, levels of TBARS in various tissues of *O. niloticus* were quite different, ranging from around 0.5 nmol/g wet weight in muscle tissue up to 4.5 nmol/g wet weight in brain samples (Figure 29 A-C). Only TBARS levels found in brain tissue (Figure 29 A) showed the tendency to increase with increasing DCF concentrations and TBARS level of $4x10^{-8}$ M DCF was statistically significant higher than in the control group. No effect on TBARS levels in gills and muscles was found.

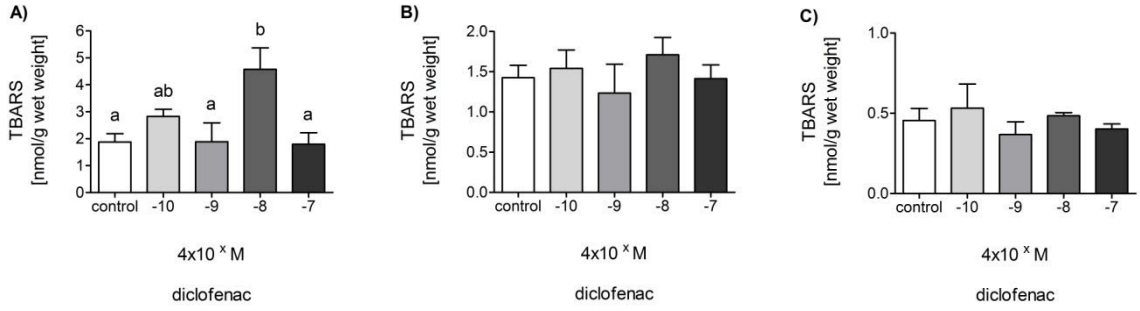


Figure 29. Levels of thiobarbituric acid reactive substances (TBARS) in (A) brain, (B) gills and (C) muscles of *Oreochromis niloticus* after 80 days post-hatch of diclofenac exposure (mean and SD) (n=5 or 6). Letters indicate statistically significant differences between treatment groups (Tukey´s Multiple Comparison test, $p < 0.05$).

3.2.2 Metoprolol

3.2.2.1 MTP analytics

MTP was not detectable in the control treatment. The effective MTP concentrations in the test water samples of the treatment concentrations were in the range of 100-112 % of the nominal values during the exposure experiment (Table 15). Again, according to OECD test guidelines 80 to 120 % of nominal are required and therefore, measured values are considered to be reliable.

Table 15. Metoprolol exposure experiment. Nominal concentrations and mean ± standard deviation (SD) of the actual exposure concentrations [µg L^{-1}] during the experiment, including percentage of the nominal concentrations. n.d.: not detectable.

Treatment [M]	**Nominal concentration [µg L^{-1}]**	**Mean measured concentration ± SD [µg L^{-1}]**	**% of nominal concentration ± SD**
control	0	n.d.	n.d.
$4x10^{-10}$	0.11	0.12 ± 0.03	110 ± 23
$4x10^{-9}$	1.07	1.20 ± 0.24	116 ± 22
$4x10^{-8}$	10.69	11.61 ± 3.17	109 ± 30
$4x10^{-7}$	106.94	116.86 ± 40.00	115 ± 37

3.2.2.2 Hatching

Hatching of *O. niloticus* larvae was completed after four days of exposure. The mean hatching rate of the control group was 99 ± 3 % (mean ± SD). Hatching success was not statistically significant affected by MTP exposure. For all treatment groups mean hatching success was almost equal to the control group, ranging from 97 ± 6 % to 99 ± 2 % (mean ± SD) (Figure 30).

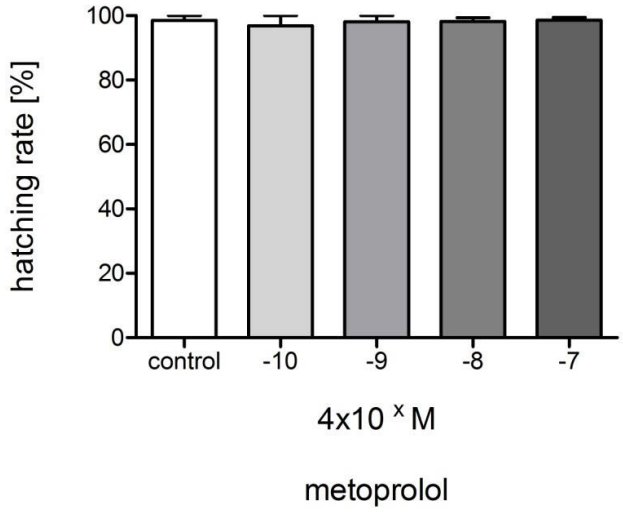


Figure 30. Hatching rates [%] of *Oreochromis niloticus* exposed to metoprolol (mean and SD). Data was analyzed using Kruskal-Wallis test revealing no statistical significant differences between treatment groups.

3.2.2.3 Survival

Survival of *O. niloticus* was slightly reduced when exposed to MTP but not significantly affected (Figure 31). Mean survival of the control fish throughout the experiment was 72 ± 8 % (mean ± SD), while the treatment groups ranged between 57 ± 18 % to 70 ± 8 % (mean ± SD). Almost no fish died until 8 dph and mortality was almost equal after 30 and 80 dph of MTP exposure (Figure 32).

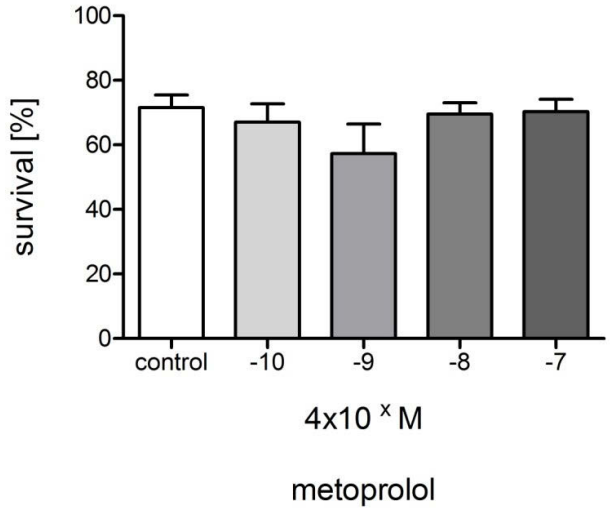


Figure 31. Survival [%] of *Oreochromis niloticus* exposed to metoprolol for 80 days post-hatch (mean and SD). Data was analyzed using Kruskal-Wallis test revealing no statistical significant differences between treatment groups.

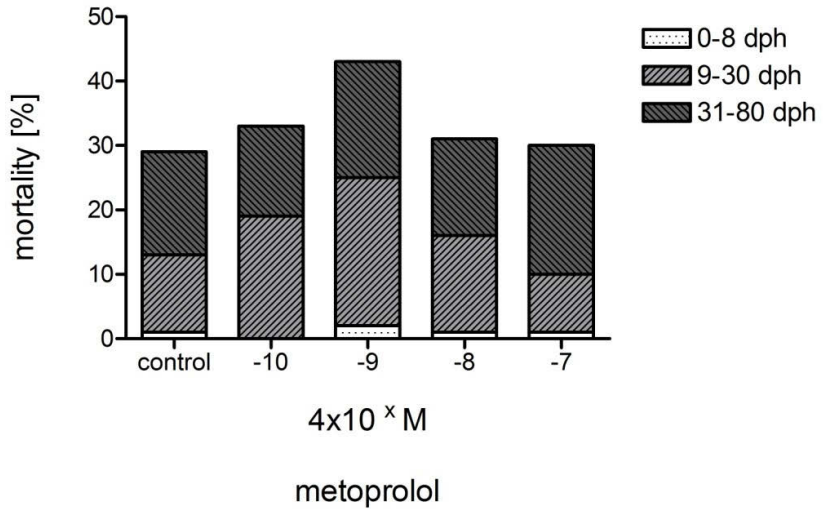


Figure 32. Cumulative mortality [%] of *Oreochromis niloticus* exposed to metoprolol for various time phases until 80 days post-hatch (dph).

3.2.2.4 Growth

After 8 dph of MTP exposure, fish wet weight was not impaired (Figure 33 A). After 30 and 80 dph, wet weight was reduced almost dose-dependently (Figure 33 B/C). A MTP concentration of $4x10^{-10}$ M caused a reduction in wet weight of only 4 % compared to the control group but the other treatment groups ranged between 87-67 % of the control group.

After 80 dph, wet weight was slightly greater when fish were exposed to $4x10^{-10}$ M MTP (108 % of control group) or equal when exposed to $4x10^{-9}$ M MTP. At higher MTP concentrations wet weight ranged between 84 - 82 % of the control group.

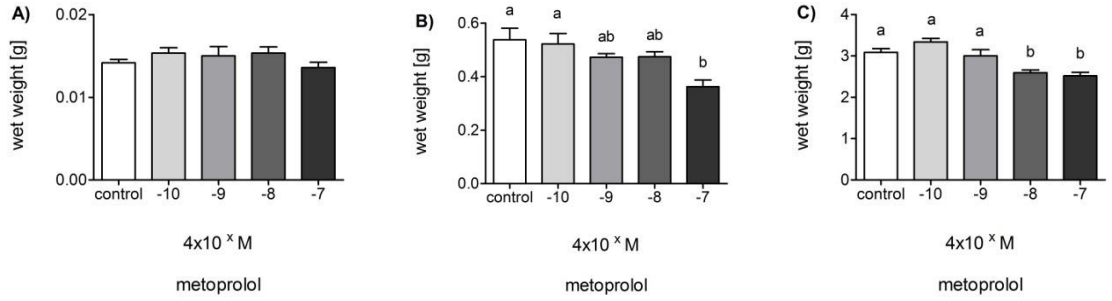


Figure 33. Wet weight [g] of *Oreochromis niloticus* after (A) 8, (B) 30 and (C) 80 days post-hatch of metoprolol exposure, respectively. The arithmetic means and SD were calculated from the mean values of the four tank replicates per treatment (n=11 or 12 at all sampling points). Letters indicate statistically significant differences between treatments (Tukey´s Multiple Comparison test, $p < 0.05$).

At all sampling points, fish length was slightly affected by MTP exposure (Figure 34). After 8 dph (Figure 34 A), length of exposed fish ranged between 103-98 % of the control group. A statistically significant difference only occurred between $4x10^{-10}$ M and $4x10^{-7}$ M MTP. After 30 dph (Figure 34 B), length was reduced dose-dependently, ranging from 98-88 % of the control group. Differences were statistically significant comparing the control group and the two lowest MTP treatment groups with the highest MTP concentration. After 80 dph (Figure 34 C), $4x10^{-8}$ M MTP caused a statistically significant reduction of fish length compared to the control. Overall, length ranged between 103 - 94 % of the control group.

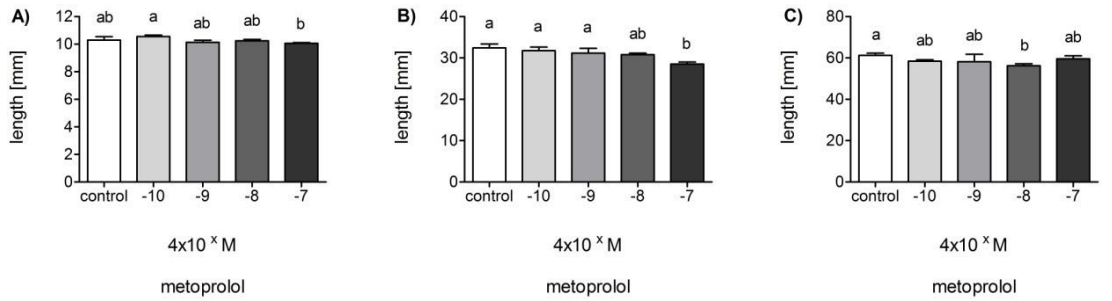


Figure 34. Length [mm] of *Oreochromis niloticus* after (A) 8, (B) 30 and (C) 80 days post-hatch of metoprolol exposure, respectively. The arithmetic means and SD were calculated from the mean values of the four tank replicates per treatment (n=11 or 12 at all sampling points). Letters indicate statistically significant differences between treatments (Tukey´s Multiple Comparison test, $p < 0.05$).

Based on the individual length and wet weight, the condition index (CI) was calculated (Table 16). Differences between treatment groups were found after 80 dph of MTP exposure. Fish exposed to $4x10^{-10}$ M MTP had the highest CI, statistically significant greater than fish exposed to $4x10^{-7}$ M MTP.

Table 16. Condition index [g/cm³] of *Oreochromis niloticus* exposed to metoprolol for 8, 30 and 80 days post-hatch (dph), respectively. Means and standard deviation (SD) are presented (n=11 or 12 at all sampling points). Letters indicate statistically significant differences between treatments (Dunn´s Multiple Comparison test).

	8 dph	**30 dph**	**80 dph**
control	1.34 ± 0.34	1.54 ± 0.06	1.38 ± 0.40 ab
$4x10^{-10}$ M	1.31 ± 0.18	1.6 ± 0.07	1.7 ± 0.31 a
$4x10^{-9}$ M	1.44 ± 0.12	1.55 ± 0.10	1.54 ± 0.28 ab
$4x10^{-8}$ M	1.44 ± 0.3	1.62 ± 0.09	1.49 ± 0.33 ab
$4x10^{-7}$ M	1.33 ± 0.22	1.53 ± 0.13	1.23 ± 0.34 b

Effects of MTP exposure on the growth axis were partly analyzed, namely by gene expression analysis of growth hormone (GH, Figure 35 A) and insulin-like growth factor I (IGF-I, Figure 35 B). No statistically significant changes in gene expression were found. Besides exposure to $4x10^{-10}$ M MTP, GH mRNA expression had a little tendency to decrease with increasing MTP concentrations. IGF-I gene expression showed no trend at all.

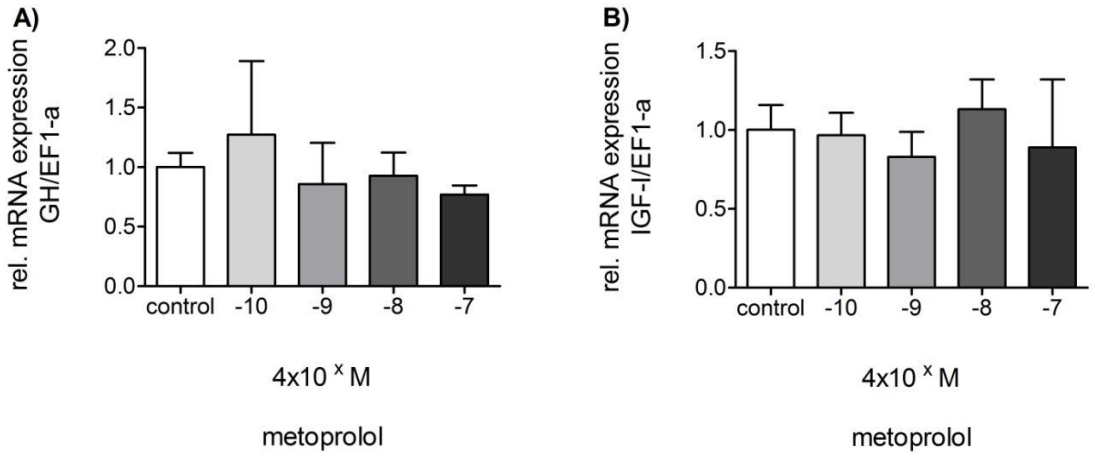


Figure 35. Relative mRNA expression of (A) growth hormone (GH) and (B) insulin-like growth factor I (IGF-I) in pituitary and liver, respectively, of *Oreochromis niloticus* after 80 days post-hatch of metoprolol exposure. Results (mean and SD) are expressed relative to control values and normalized to elongation factor 1-α (EF1-a) (n=7 or 8). Data was analyzed using one way ANOVA revealing no statistically significant differences between treatment groups.

3.2.2.5 Hepatosomatic index

The HSI did not differ statistically significant due to MTP exposure, neither 30 nor 80 dph (Table 17). After 80 dph, the HSI tended to increase with higher MTP concentrations. The HSI due to $4x10^{-7}$ M MTP was about one third higher compared to the control group.

Table 17. Hepatosomatic index [%] of *Oreochromis niloticus* exposed to metoprolol for 30 and 80 days post-hatch (dph). Means and standard deviation (SD) are presented (n=10 to 12). Data was analyzed using one way ANOVA revealing no statistical significant differences between treatment groups.

	30 dph	**80 dph**
control	2.98 ± 0.21	0.66 ±0.09
$4x10^{-10}$ M	3.41 ± 0.35	0.75 ± 0.04
$4x10^{-9}$ M	3.20 ± 0.29	0.79 ± 0.13
$4x10^{-8}$ M	2.98 ± 0.58	0.72 ± 0.13
$4x10^{-7}$ M	2.81 ± 0.55	0.89 ± 0.05

3.2.2.6 Histology

Using light microscopic examinations of gills of *O. niloticus* after 80 dph of exposure to MTP, only few histopathological alterations could be observed. Overall, MTP exposure had no effect on the total number of SLs with alterations (Figure 36). In the control group 54 % of SLs were affected. In the MTP treatment groups between 40 - 56 % of SLs were affected. In total, hyperplasia and hypertrophy of chloride cells occurred most often. Moderate alterations were only found occasionally but severe alterations were almost not found at all. Compared to the control group, epithelial lifting occurred significantly less after $4x10^{-9}$ M MTP exposure (Figure 37 A and Figure 38 D) but no difference between the other treatment groups were found. Hypertrophy was found more often in the MTP treatment groups, but differences were not statistically significant (Figure 37 B). Hyperplasia of epithelial cells tended to be most abundant in the control group as well as $4x10^{-10}$ M MTP (Figure 37 C and Figure 38 B), but again no statistically significant effect was found. In contrast, proliferation of cells occurred dose-dependently (Figure 37 D) with statistically significant differences to the control group for the three highest MTP concentrations. Leucocytes were found most often in the $4x10^{-7}$ M MTP treatment, being significant compared to the control group (Figure 37 E and Figure 38 C).

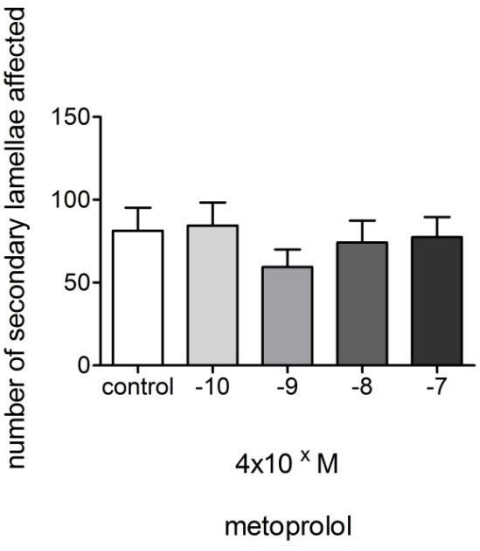


Figure 36. Total number of secondary lamellae (and SD) with histopathological alterations in gills of *Oreochromis niloticus* after 80 days post-hatch of metoprolol exposure (n=8). Data was analyzed using one way ANOVA revealing no statistical significant differences between treatment groups.

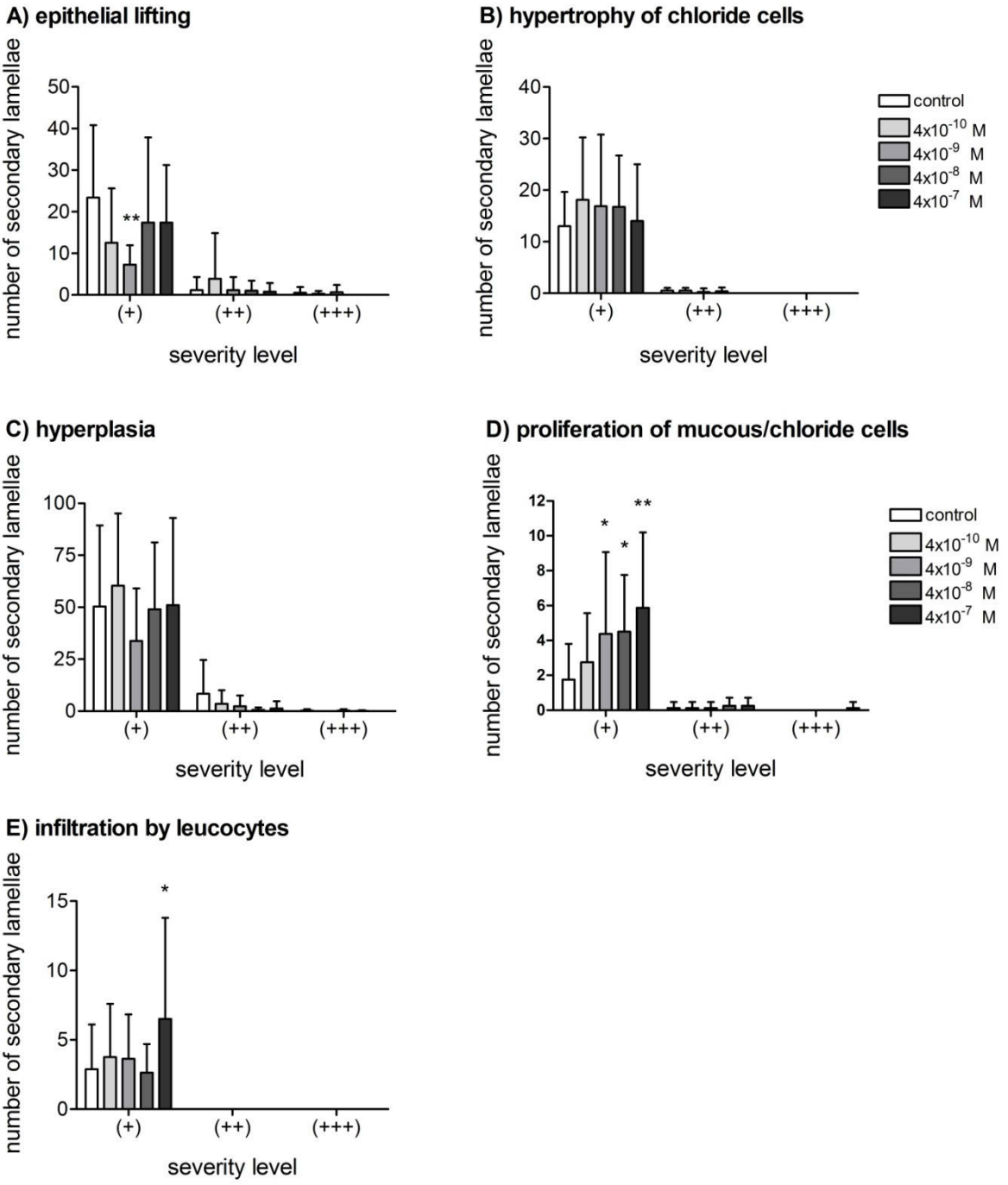


Figure 37. Mean number of secondary lamellae (out of 150) and SD in gills of *Oreochromis niloticus* with the following findings after 80 days post-hatch of metoprolol exposure: (A) epithelial lifting, (B) hypertrophy of chloride cells, (C) hyperplasia, (D) proliferation of mucous/chloride cells, (E) infiltration by leucocytes. Alterations are categorized: (+) mild, (++) moderate, (+++) severe. Note varying scales. Asterisks indicate statistically significant differences to control within one severity level of lamellar damage (Bonferroni post test *$p < 0.05$, **$p < 0.01$).

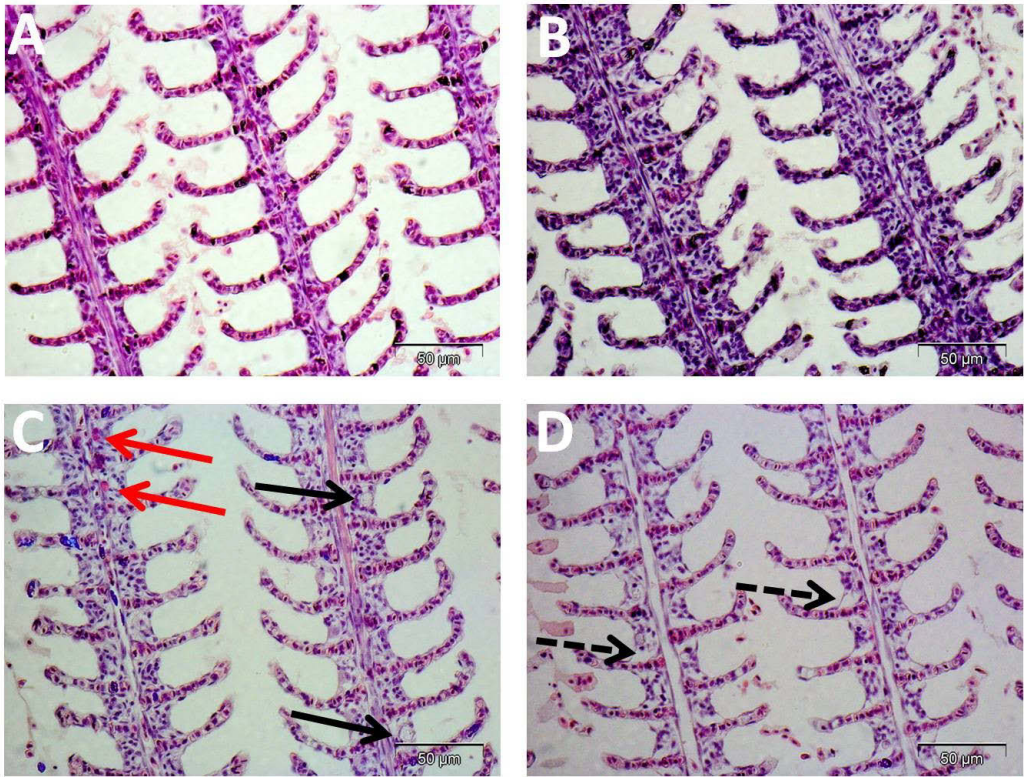


Figure 38. Gill histology of *Oreochromis niloticus* after 80 days post-hatch under experimental conditions. (A) control, (B-D) exposure to $4x10^{-7}$ M metoprolol. Histopathological alterations: (B) hyperplasia, (C) infiltrations by leucocytes (red arrows) and hypertrophy of chloride/mucus cells (solid black arrows) and (D) epithelial lifting (dotted black arrows). Original magnification 400 x, scale bar corresponds to 50 µm, sections of 4 µm thickness and stained by hematoxylin and eosin method.

3.2.2.7 Gene expression of pituitary gonadotropins and hepatic vitellogenin

Gene expression patterns of both pituitary gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH) were analyzed to gain information about the influence of MTP exposure on the hypothalamus-pituitary-gonad axis (HPG axis, Figure 39). Expression patterns were similar for both hormones. Exposure to $4x10^{-10}$ M MTP caused the highest mRNA expression (LH: 1.9-fold, FSH: 1.5-fold compared to control). All other MTP treatment concentrations possessed a lower gene expression compared to the control as well as the lowest MTP treatment group. Statistically significant differences were not found compared to the control group but comparing $4x10^{-10}$ M MTP to $4x10^{-8}$ M and $4x10^{-7}$ M considering LH (Figure 39 A) and comparing $4x10^{-10}$ M MTP to $4x10^{-9}$ M and $4x10^{-7}$ M MTP considering FSH (Figure 39 B).

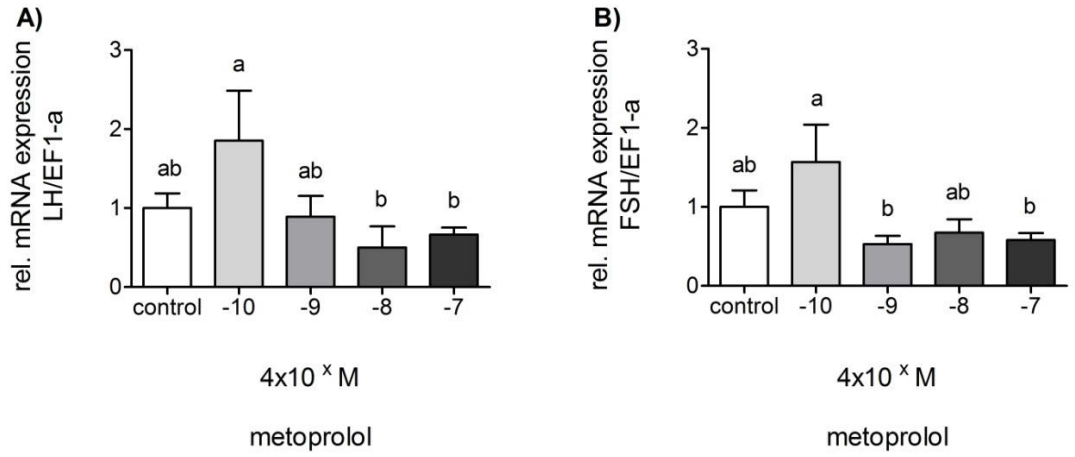


Figure 39. Relative mRNA expression of (A) luteinizing hormone (LH) and (B) follicle stimulating hormone (FSH) in the pituitary of *Oreochromis niloticus* after 80 days post-hatch of metoprolol exposure. Results (mean and SD) are expressed relative to control values and normalized to elongation factor 1-α (EF1-a) (n=6 to 8). Letters indicate statistically significant differences between treatments (Tukey´s Multiple Comparison, $p < 0.05$).

Relative mRNA expression of VTG (Figure 40) increased only statistically significant due to exposure to $4x10^{-7}$ M MTP. The induction was about 2.9-fold. The lower MTP concentrations had almost no effect on VTG gene expression. $4x10^{-10}$ M MTP caused no change in expression pattern, while $4x10^{-9}$ M and $4x10^{-8}$ M MTP induced the expression level 1.4- and 1.3-fold, respectively.

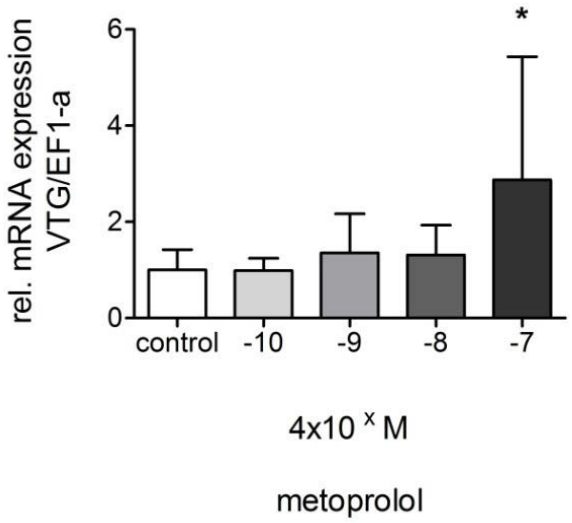


Figure 40. Relative mRNA expression of vitellogenin (VTG) in the liver of *Oreochromis niloticus* after 80 days post-hatch of metoprolol exposure. Results (mean and SD) are expressed relative to control values and normalized to elongation factor 1-α (EF1-a) (n=7). Asterisks indicate statistically significant differences to control (Tukey´s Multiple Comparison test *$p<0.05$).

3.2.2.8 Biotransformation

Relative mRNA expression of three key enzymes of the biotransformation pathways was analyzed at all sampling points, using whole larvae after 8 dph and liver samples after 30 and 80 dph, respectively. Gene expression patterns were not significantly changed due to MTP exposure at any sampling point. Exemplary, gene expression levels after 80 dph of MTP exposure are shown (Figure 41). MTP increased CYP1A gene expression with the highest induction due to $4x10^{-7}$ M MTP (3.6-fold, Figure 41 A). Relative mRNA of GST was almost not altered due to MTP exposure at all (Figure 41 B), while MDRP gene expression had higher values due to MTP exposure, ranging from almost 2-fold to 2.7-fold with increasing MTP concentrations (Figure 41 C).

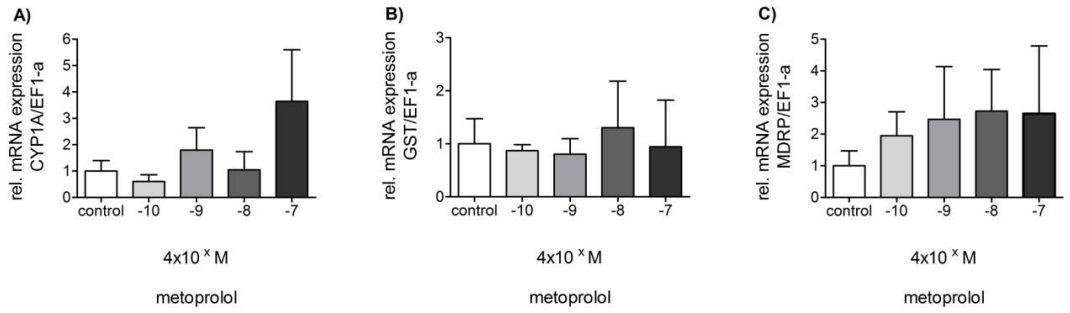


Figure 41. Relative mRNA expression of (A) cytochrome P4501A monooxygenase (CYP1A), (B) glutathione-S-transferase (GST) and (C) multidrug resistance protein (MDRP) in the liver of *Oreochromis niloticus* after 80 dph of metoprolol exposure. Results (mean and SD) are expressed relative to control values and normalized to elongation factor 1-α (EF1-a) (n=6 or 7). Data was analyzed using Kruskal-Wallis test revealing no statistically significant differences between treatment groups.

Additionally, CYP1A and GST gene expression was analyzed in gills of *O. niloticus* after 80 dph of MTP exposure (Figure 42). Except for $4x10^{-9}$ M MTP, CYP1A gene expression tended to increase with increasing MTP concentrations, although being statistically not significant (Figure 42 A). The decrease at $4x10^{-9}$ M MTP was statistically significant compared to $4x10^{-10}$ M MTP. No effect of MTP exposure on GST gene expression was found (Figure 42 B).

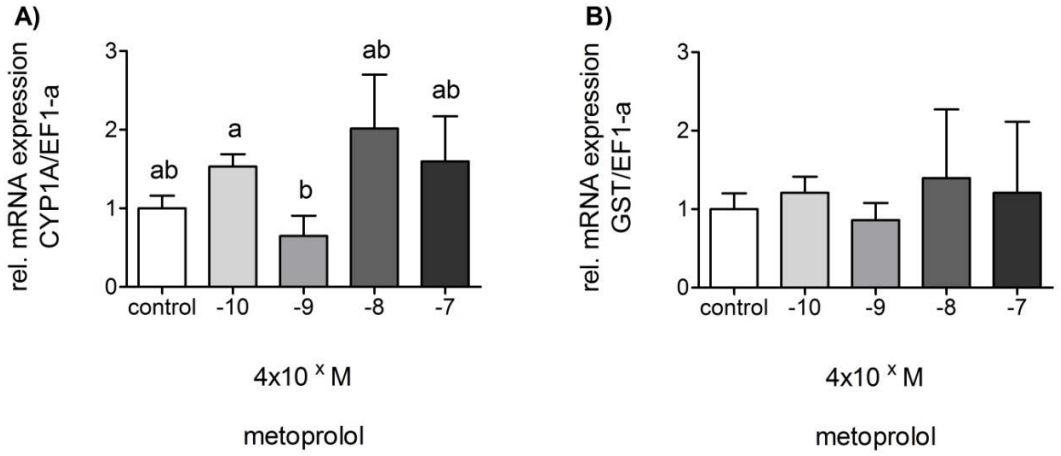


Figure 42. Relative mRNA expression of (A) cytochrome P4501A monooxygenase (CYP1A) and (B) glutathione-S-transferase (GST) in the gills of *Oreochromis niloticus* after 80 dph of exposure to metoprolol. Results (mean and SD) are expressed relative to control values and normalized to elongation factor 1-α (EF1-a) (n=5 or 6). Letters indicate statistically significant differences between treatments (Dunn´s Multiple Comparison test, $p<0.05$).

3.2.2.9 Oxidative stress

In brain tissue of *O. niloticus*, TBARS levels were statistically significant higher after exposure to $4x10^{-10}$ M and $4x10^{-8}$ M MTP compared to the control group (Figure 43 A). The two other MTP treatment concentrations had no effect. The same pattern was found in muscle tissue (Figure 43 C). In gills, no effect of MTP exposure on TBARS levels occurred but $4x10^{-7}$ M MTP caused a non-significant increase of about 2-fold compared to all other groups (Figure 43 B).

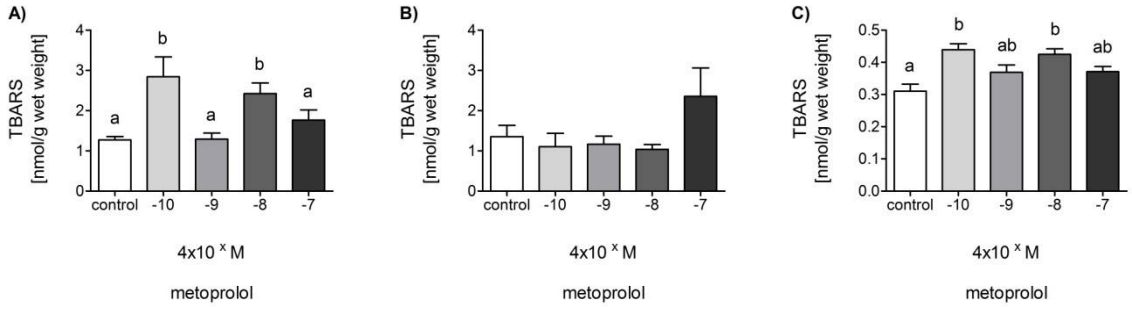


Figure 43. Levels of thiobarbituric acid reactive substances (TBARS) in (A) brain, (B) gills and (C) muscles of *Oreochromis niloticus* after 80 days post-hatch of metoprolol exposure (mean and SD) (n=6). Letters indicate statistically significant differences between treatment groups (Tukey´s Multiple Comparison test).

4 Discussion

Pharmaceuticals of many different therapeutic classes are frequently found in the aquatic environment and many of them remain biologically active, altering physiology and impairing health of non-target organisms such as fish. NSAIDs and ß-blockers are among those highly consumed substances and hence, of high environmental relevance. Mainly based on acute toxicity studies, DCF has often been classified as a threat to aquatic wildlife. Few studies exist that provide evidence that MTP also cause changes in fish physiology. Long-term exposure studies in the environmentally relevant concentration range are still rare for most pharmaceuticals. The goal of the present work was to gather information to fill in that gap and to assess physiological effects of DCF and MTP on (developing) fish with focus on population relevant endpoints, detoxification mechanisms and the potential to disrupt the endocrine homeostasis with emphasis on the reproductive system. For reasons of clarity, results are initially discussed separately for the *in vitro* (both substances together) and *in vivo* (substances separated) studies and finally integrated in a short comparative section.

4.1 *In vitro* experiments

Primary hepatocytes are considered to be the closest available *in vitro* experimental model system in terms of normal fish liver physiology (Schmid et al. 2000). They possess all benefits of intact cells such as functional organelles and enzyme interactions (Segner 1998) and are therefore a valid tool to assess detoxification biomarkers and provide initial information on the endocrine activity of test substances (Kloas et al. 1999; Navas and Segner 2006).

4.1.1 Detoxification

Direct effects on the biotransformation in primary hepatocytes of *O. niloticus* were investigated, since the detoxification pathway is mainly located in the liver. It typically consists of three major phases and gene expression patterns of key enzymes of these three phases were analyzed.

4.1.1.1 Cytochrome P4501A monooxygenase

DCF induced biotransformation phase I enzyme CYP1A in primary hepatocytes of *O. niloticus* already at environmentally relevant concentrations of $4x10^{-9}$ M (about 1 µg L^{-1}).

However, higher concentrations ($4x10^{-8}$ M and $4x10^{-7}$ M) did not lead to a statistically significant greater induction compared to $4x10^{-9}$ M. These results suggest that CYP1A is moderately involved in DCF metabolism in Nile tilapia. In agreement, after exposure to 1 µg L^{-1} DCF, Hong et al. (2007) found CYP1A expression to be 9.3-fold increased (*in vivo*) in the liver of Japanese medaka (*O. latipes*). In contrast, Laville et al. (2004) analyzed the effects of different pharmaceuticals including DCF in primary rainbow trout (*O. mykiss*) hepatocytes and fish cell lines by determining cell viability and induction of CYP1A catalytic activity (EROD). They found an inhibition of EROD at a concentration of $3.6x10^{-5}$ M DCF. Triebskorn et al. (2004) demonstrated that *in vivo* exposure to environmentally relevant concentrations of DCF led to cellular reactions in various tissues of rainbow trout after exposure to < 100 µg L^{-1} (about $4x10^{-7}$ M). These findings have been interpreted as activated stress status, and modifications of the structure of cell organelles as intensified need for detoxification capacities. According to Bort et al. (1999) the mechanisms of DCF cytotoxicity are not fully understood but there is evidence that CYP-mediated metabolism is also involved when human and rat hepatocytes experience acute toxicity.

Based on these findings, DCF seems to be cytotoxic for Nile tilapia hepatocytes already at environmentally relevant concentrations. CYP1A is an accepted indicator of cellular toxicity and an increase in hepatic CYP1A expression is consistent with the role of the liver in xenobiotic metabolism and excretion (Sarasquete and Segner 2000). In comparison to Hong et al. (2007), the *in vitro* response presented in this study resembles the *in vivo* situation found by the authors in terms of sensitivity of the different models, although the increase in CYP1A expression was higher in the *in vivo* system. As stated by Ferreira et al. (2014), concentration dependent induction of CYP1A shows that the *in vitro* approach reflects physiologically relevant *in vivo* situations and should be considered as a reliable tool to assess phase I biotransformation enzymes.

In comparison to DCF, induction of CYP1A gene expression levels after MTP exposure was statistically significant only at a concentration 100-fold higher ($4x10^{-7}$ M) than the lowest concentration. It seems that Nile tilapia is less sensitive towards exposure to MTP compared to DCF considering the effects on CYP1A gene expression. Triebskorn et al. (2007) found MTP to cause ultrastructural effects, such as collapse of cellular compartmentation and glycogen reduction in rainbow trout liver exposed to 1 µg L^{-1} MTP. The authors concluded that these structural reactions might indicate the activation of enzymes equivalent to

mammalian cytochromes of the CYP family and therefore an induction of biotransformation in the fish liver. In humans (Belpaire et al. 1998) and mussels (Contardo-Jara et al. 2010) there is good evidence that MTP biotransformation occurs via CYP enzymes as well. Another non-selective ß-blocker (propranolol) was also classified as a CYP1A inducer based on a study using trout hepatocytes (Laville et al. 2004).

4.1.1.2 Glutathione-S-transferase

Like CYP1A, biotransformation phase II enzyme GST was induced due to DCF exposure at all concentrations tested. Again, even the environmentally relevant concentration up-regulated GST statistically significant compared to the control group. This increase in GST mRNA after DCF exposure indicates that phase II biotransformation occurs, most likely further metabolizing CYP1A activated DCF. Stepanova et al. (2013) found DCF to change activity of GST only at concentrations of 3 mg L^{-1} ($1x10^{-5}$ M) in early life stages of carp (*Cyprinus carpio*) after 30 days of *in vivo* exposure.

Studies of the effects of MTP on GST gene expression are scarce. In mussels (*Dreissena polymorpha*), phase II enzymes seem to be involved in biotransformation processes of MTP. Contardo-Jara et al. (2010) found an increase of piGST mRNA expression after four to seven days exposure to $2x10^{-7}$ M and $2x10^{-6}$ M MTP and concluded that GST mRNA induction is a valid, sensitive tool to investigate the effects of exposure of non-target aquatic organisms to environmentally relevant concentrations of PhACs (around 1 µg L^{-1}). Apparently, no other *in vitro* data on the effects of DCF and MTP on expression levels of GST in (Nile tilapia) primary hepatocytes is available.

4.1.1.3 Multidrug resistance protein

DCF exposure induced MDRP gene expression levels up to 3-fold compared to the control group. Expression levels tend to increase with elevating concentrations (1.9 to 3.0-fold) but, due to high SDs at the higher concentrations, this difference only reached statistical significance at a concentration of $4x10^{-9}$ M. Various authors found evidence that xenobiotics induce ABC transporter gene expression in fish, for example due to exposure to pharmaceuticals (Corcoran et al. 2012), polycyclic aromatic hydrocarbons (Paetzold et al. 2009) or heavy metals (Long et al. 2011). Up-regulation of gene expression of ABC transporters as found here after DCF exposure could be a response to the presence of DCF

and/or its metabolites but further studies would be necessary to confirm this hypothesis at the post-transcriptional and protein level.

Activated and conjugated drugs and their metabolites need to be eliminated by the organisms. Therefore, it makes sense that an increase of phase I and II enzyme gene expression occurs in parallel to an increase of phase III metabolism. DCF and its metabolites seem to be at least partly eliminated by MDRP. With respect to MTP, it is likely that other enzymes of the ABC superfamily catalyze excretion since the induction of MDRP mRNA was not statistically significant different from the control (although expression level was up to 2.7-fold higher). In mussels, exposure to ß-blockers (MTP, propranolol) induced P-gp mRNA expression (Contardo-Jara et al. 2010; Fabbri et al. 2009). P-gp is known to transport relatively non-specific xenobiotic compounds and their metabolites (Smital et al. 2003). However, Caminada et al. (2008) found MTP and propranolol to have no or an inhibitory effect on P-gps in the fish cell line PLHC-1.

According to Leslie et al. (2001), MDRP supports the maintenance of the cellular redox status and therefore the cellular GSH homeostasis by transporting glutathione (GSH) or glutathione disulphide (GSSG). GSH has many important roles in the protection of cells from oxidative stress. Since none of the substances tested caused a huge increase in MDRP mRNA, it seems unlikely that the substances cause oxidative stress in Nile tilapia hepatocytes.

4.1.2 Vitellogenin

VTG induction can be measured in fish hepatocytes *in vitro* and has been suggested as a useful tool for screening of endocrine disruption potential concerning estrogenicity (Navas and Segner 2006). EE2 was included in the experimental design as a substance well-known to cause estrogenic action by binding to estrogen receptors. The concentration of $1x10^{-6}$ M induced VTG gene expression statistically significant compared to the control group. EE2 exposure led to a 2.0-fold increase in VTG gene expression. The effect therefore ranged between a DCF exposure of $4x10^{-8}$ M (1.4-fold) and $4x10^{-7}$ M (3.0-fold) and caused a higher induction than any of the MTP concentrations.

VTG gene expression was up-regulated in a dose-dependent manner after exposure to DCF, although only the lowest and highest concentrations were statistically significant different from the control group. Many pharmaceuticals are known to have the potential to promote

estrogenic activities (Kloas et al. 2009) and different studies provide evidence that DCF is one of them. *In vivo*, Hong et al. (2007) found an increase of VTG gene expression in male medaka of 46.6-fold at 1 µg L^{-1} DCF and of 179.5-fold at 8 mg L^{-1} DCF after four days of exposure. The authors concluded that DCF has an estrogenic potential, likely to cause endocrine disruption in fish.

MTP induced VTG mRNA between 1.2 and 1.6-fold, while the difference was only statistically significant at $4x10^{-8}$ M. Massarsky et al. (2011) reviewed the potential effects of human ß-blockers on aquatic organisms and found species-specific endocrine disruption. Huggett et al. (2002) showed that plasma E2 was significantly increased in male medaka after two weeks of propranolol exposure at all concentrations tested.

4.2 *In vivo* DCF exposure

Conducting an *in vivo* experiment is one of the most comprehensive approaches to mimic natural conditions in the laboratory. Certain advantages appear since, for example, control groups can be included in the experimental design and physico-chemical conditions can be easily controlled. Nonetheless, laboratory experiments are always run in rather artificial environments.

4.2.1 General and morphological parameters

Two main population relevant endpoints, namely hatching success and survival after 80 dph, were not impaired by DCF exposure at any treatment concentration ($4x10^{-10}$ M to $4x10^{-7}$ M). In general, *O. niloticus* was found to be transiently most sensitive to exposure treatment between 8 to 30 dph of exposure, thus in the passage from yolk sac feeding to free swimming and free feeding state. Differences between the sensitivity of the embryonic developmental stages of fish and juvenile/adult individuals of the same species could be due to the improper developed enzymatic systems, differences in metabolism pathways or different processes of absorption of substances into organisms (van Leeuwen et al. 1985) and therefore emphasize the validity of chronic exposure studies, covering different developmental stages.

Ibuprofen, another NSAID, caused a statistically significant decrease in egg production, reduced hatchability and increased embryo mortality in zebrafish (*Danio rerio*) exposed to 1-10 µg L^{-1} for only 21 days. As emphasized by Overturf et al. (2015), different fish species possess different sensitivities to pharmaceutical exposure. After 8 and 30 dph, growth (neither

wet weight nor length) was not affected by DCF exposure, but after 80 dph there was a slight reduction in fish growth due to exposure to $4x10^{-8}$ M DCF. IGF-I gene expression showed the same tendency to decrease with increasing DCF concentrations, although not confirmed by statistical evaluation. Additionally, the CI was reduced after 30 dph exposure to $4x10^{-8}$ M DCF. However, these results showed no distinct dose-dependency. The calculated HSI showed the tendency to increase dose-dependently after 30 dph but no remaining trend was found after 80 dph. Thus, one may speculate that the juvenile tilapia after 80 dph might somewhat better cope with DCF exposure compared to 30 dph by having a higher developed detoxification system, which might need some energy diminishing in turn a bit growth. Lee et al. (2011) exposed Japanese medaka for three month to concentrations of 1-10,000 µg L^{-1} DCF and did not find any change in HSI as in this study after 80 dph. In contrast, Guiloski et al. (2015) found a reduction in fish liver size, revealing a reduced HSI, after six weeks of trophic exposure to 0.2 µg kg^{-1} DCF in Tiger fish *(Hoplias malabaricus*). However, higher doses (2 and 20 µg kg^{-1} DCF) had no statistically significant effect though. The authors concluded that the reduced HSI could be due to a depletion of glycogen in the hepatocytes as found by Triebskorn et al. (2004) in rainbow trout but trophic exposure is not the natural exposure route of DCF for fishes and thus is hardly comparable to aqueous exposure. Hepatocytes growth can be affected by changes in the function of these cells when exposed to NSAIDs (Flippin et al. 2007). Ultrastructural examination showed that DCF induced cellular reactions indicating an activated hepatic metabolism in the trout liver already after exposure to 1 µg L^{-1} (Triebskorn et al. 2004). Furthermore, mRNA expression of GH tended to increase with elevating DCF concentrations. Pickering et al. (1991) found circulating levels of GH in rainbow trout after chronic stress of low oxygen levels to increase significantly and linked these to an elevation of plasma cortisol in the same fish. GH is considered to be a stress-related hormone in fish and exposure to environmentally important pollutants, including xenoestrogens, can affect GH mediated mechanisms (Deane and Woo 2009). Hence, the increase in GH found in this study could be an indicator for stress induction in Nile tilapia due to DCF exposure but confirmation by further analyses of e.g. plasma cortisol levels is necessary to confirm the non-significant finding.

4.2.2 Histopathological alterations

The impacts of pharmaceutical exposures on gill physiology are of special interest, since adverse alterations of gills most likely affect oxygen supply and hence all biochemical reactions depending on oxygen (Hoeger et al. 2005). Nonetheless, technical guidelines for the

preparation and histopathological evaluation of fish organs other than gonads, that would increase the comparability of different study results, are still missing (Memmert et al. 2013) but of urgent need as shown by Wolf et al. (2014). The authors re-evaluated three previously published studies of DCF exposure in trout (Hoeger et al. 2005; Mehinto et al. 2010; Memmert et al. 2013) concerning their histopathological outcomes. They applied a pathology peer review/pathology working group model known from mammalian toxicologic pathology. Briefly, this means that besides the study pathologist another, reviewing pathologist, evaluates the histopathological alterations while knowing the outcome of the first evaluation (for more details see Wolf et al. 2014). Aim of this re-evaluation was to reconcile the previous mentioned differences in study results and to establish an appropriate NOEC of DCF based on histopathology which was set to be >320 µg L^{-1} afterwards. Unfortunately, the study by Schwaiger et al. (2004) could not be re-evaluated due to missing material and overall, the quality of the re-evaluated material was judged to be rather fair. Based on the re-evaluation, histopathological effects of environmentally relevant concentrations of DCF on organs of trout species seem unlikely.

Nonetheless, histopathological changes of SLs in *O. niloticus* gills after DCF exposure were found and mainly characterized as mild, only epithelial lifting also occurred notably in a severe manner. Overall, epithelial lifting (including formation of edema) and proliferation of epithelial cells (hyperplasia) were the most dominant findings. As stated by Wolf et al. (2015), especially epithelial lifting is susceptible to occur as an artifact during sampling and fixation process. To reduce handling biases, it was suggested to pay special attention to the procedures that take place at or around the time of death and to use Bouin´s solution for fixation. In the present study, experimental fish of the control group were treated the same way than fish of the DCF exposure groups and Bouin´s solution was used. Therefore, provisions were made against the occurrence of artifact. The rather severe occurrence of epithelial lifting is in agreement with other study results (Schwaiger et al. 2004; Triebskorn et al. 2004; Monteiro et al. 2008) and indicates an impaired osmoregulation due to damaged epithelia (Wood 2001), further confirmed by the occurrence of hypertrophy in chloride cells. Proliferation of epithelial cells is a common result of chronic sub-lethal xenobiotic exposure (Mallat 1985; Wood 2001) and in combination with the proliferation of chloride and mucous cells, seen as an adaptation mechanisms to reduce the uptake by increasing the blood-water diffusion distance (Wood 2001). Unexpectedly, proliferation of mucous/chloride cells was found more often in the control group than in the two lowest exposure treatments.

After 21 days exposure to 50 µg L^{-1} DCF, Hoeger et al. (2005) found a statistically significant increase of lamellar clubbing, while epithelial cell hyperplasia, thickened lamellar tips and secondary lamellar fusion were only occasionally observed. Immunohistology revealed an increase of granulocyte numbers in the primary gill filaments. According to the authors, the infiltration of granulocytes may support the presence of inflammatory processes, possibly in response to the damage of vascular endothelium. Leucocyte infiltration was observed rather seldom in the control group and after $4x10^{-7}$ M DCF exposure. Only one sample of the highest exposure group had leucocytes present (with the highest number of leucocytes found at all), while all other replicates did not show this alteration. Almost all samples of the other exposure groups showed leucocyte infiltration though. These results could reflect the anti-inflammatory mode of action of DCF in fish at certain exposure concentrations. Schwaiger et al. (2004) calculated a mean assessment value (MAV) based on three grades of histopathological alterations in the gills of trout and this value was already highly significantly different than the control group after 28 days exposure to 5 µg L^{-1} DCF.

The findings of Wolf et al. (2014) raise concern about the general reliability of histopathological changes published and emphasize the need of good scientific practice and documentation. In combination with the findings of this study they also shown that exposure studies should cover a variety of fish species since fish are a taxonomic diverse group adapted to different environmental conditions and therefore, it is likely that they posses different sensitivities.

Endpoints discussed so far (morphologically as well as molecular biologically) indicate only slight impacts of DCF exposure on Nile tilapia, mostly missing a clear dose-dependency. Only histopathological alterations showed clear but rather mild to moderate effects.

4.2.3 Gene expression of pituitary gonadotropins and hepatic vitellogenin

In contrast, effects of DCF exposure on reproduction related endpoints and estrogenicity were more pronounced. Gene expression of LH was reduced almost dose-dependently after 80 dph of DCF exposure. LH stimulates the testosterone production via the HPG axis (Milla et al. 2009) and a reduced expression pattern interferes with the normal sex development. Therefore, a decrease in LH gene expression can be considered as an indicator of the endocrine activity of DCF, further confirmed by findings of Fernandes et al. (2011) who demonstrated an interference with the sex steroid synthesis due to DCF exposure in common

carp (*C. carpio*). Vajda et al. (2011) exposed adult male fathead minnows (*P. promelas*) to the effluents of a wastewater treatment plant containing pharmaceuticals and found a demasculinization of the test organisms. Furthermore, a non-monotonic dose response relationship (NMDR) of VTG gene expression was found with an inverted U-shape due to DCF exposure, peaking at $4x10^{-9}$ M, which was nearly mirrored by the LH and FSH patterns, suggesting negative feedback mechanisms of DCF on pituitary gonadotropins via an estrogenic mode of action. The maximal response was observed at an intermediate dose, highlighting the general need for low-dose testing in ecotoxicological research. In case of NMDRs a safe dose determined from high doses does not guarantee safety at lower, untested doses (Vandenberg et al. 2012). In male medaka, Hong et al. (2007) found an increase of VTG gene expression of 47-fold at 1 µg L^{-1} and 180-fold at 8 mg L^{-1} DCF and in male *Xenopus laevis*, Efosa et al. (in. prep., pers. comm.) found a dose-dependent induction of VTG gene expression of about 2-fold after eight days exposure to 10^{-10} M and 10^{-8} M DCF. Furthermore, the authors could show that DCF exposure caused estrogen-like behavioral effects in male *X. laevis* and, based on these findings, concluded a light to moderate estrogenicity of DCF in amphibians. Overall, VTG gene expression analysis is a valid tool to study the endocrine disruption potential concerning estrogenicity of pharmaceuticals (Navas and Segner 2006) and taking the different studies with various organisms into account, DCF seems to possess estrogenic endocrine disruption potential. As reviewed by Overturf et al. (2015), reproductive effects of NSAIDs tend to vary between experiments and species and overall, the effects of prostaglandin inhibition in fish on reproductive success remain unclear.

4.2.4 Detoxification

Data obtained *in vitro* indicated that DCF is metabolized through a CYP1A-GST-MDRP route in *O. niloticus* primary hepatocytes (Gröner et al. 2015). These enzymes were found by different authors to be involved in the detoxification metabolism of pharmaceuticals (e.g. Hong et al. 2007; Contardo-Jara et al. 2010; Corcoran et al. 2012) and an induction was also found *in vivo* in this study. Combining VTG gene expression data with mRNA expression data of these key enzymes of the biotransformation pathway, it seems likely, that an increased metabolism and excretion (with exception for $4x10^{-7}$ M DCF) is jointly responsible for the inverted U-shaped response in VTG mRNA expression. At the higher concentrations the detoxifying biotransformation might be more efficient to metabolize and excrete DCF, which in turn might lead to a lower availability of DCF acting as an estrogenic EDC in tilapia. At $4x10^{-8}$ M DCF, MDRP gene expression was induced about 5-fold, while VTG gene

expression was reduced by almost 50 % compared to the previous concentration. No clear reason for the performances at the highest concentration ($4x10^{-7}$ M DCF) can be given, but further adverse side effects or adaptation to uptake, metabolization, and/or excretion of DCF as well as general impairment effects could be possible. Histopathological examinations of liver tissue would give further indication.

Gene expression patterns of CYP1A and GST were also analyzed in gill samples. The gills are the primary site of exposure to waterborne chemicals and hence, knowledge of their potential to metabolize xenobiotics is important to predict resulting plasma concentrations and downstream biological effects (Bartram et al. 2012). They are one main biological barrier in direct contact with environmental pollutants and known to exhibit biotransformation enzymes (Hong et al. 2007). CYP1A was not statistically significant altered by DCF exposure in gills but showed some tendency to decrease with increasing DCF concentrations. This is in contrast to findings of Hong et al. (2007) who found an induction of CYP1A in gills of Japanese medaka after exposure to 1 µg L^{-1} DCF. Mehinto (2009) also found an increase in CYP1A1 relative expression in gills of rainbow trout at 0.5 µg L^{-1} DCF, with a maximum up-regulation of less than 2-fold. On the other hand, GST was initially induced with increasing DCF concentrations (statistically significant for $4x10^{-9}$ M DCF) but the two highest concentrations showed a distinct reduction compared to the other treatment groups. These results might indicate that at lower DCF concentrations biotransformation processes in gills are increasingly active but higher DCF concentrations might already cause cellular damage as indicated by histological examinations. Unlike the liver, gills are in direct contact to the surrounding pharmaceutical concentrations and therefore, impairment might occur already at lower concentrations than in the liver. In gills, it seems that exposure to $4x10^{-8}$ M DCF reduces gill functionality already and therefore gene expression of GST was reduced.

4.2.5 Oxidative stress

As an unspecific measure for oxidative stress, TBARS levels in three different tissues were determined. Gill and muscle samples showed no differences in TBARS level after DCF exposure but in brain samples a statistically significant increase was found due to $4x10^{-8}$ M DCF. Saucedo-Vence et al. (2015) found a statistically significant increase of LPO compared to the control in gills, muscle and brain of common carp after exposure to around 7 mg L^{-1} DCF for 24 days. Comparing these values to the LPO level after four days of exposure, the authors found a decrease in muscle and brain tissue and an increase only in gill

tissue. Stepanova et al. (2013) found a statistically significant reduction in TBARS levels in whole body samples of embryos and larvae of common carp after 30 days of exposure to 0.03, 1 and 3 mg L^{-1} DCF. In zebrafish embryos, Feito et al. (2012) also found a reduction in LPO after 90 min of DCF exposure (0.03 µg L^{-1}) and the authors conclude that their results are in agreement with the protective effects of NSAIDs against oxidative stress as mentioned by Petersen et al. (2005). Taking the different results into account it seems that LPO and TBARS level evaluations are rather cautious to interpret and the single increase in TBARS level in brain tissue found in this study should not be overestimated. Based on these findings, it seems most likely that DCF at environmentally relevant concentrations does not cause oxidative stress in Nile tilapia. Gene expression analyses of enzymes involved in oxidative damage defense such as CAT or GPx would give further indication.

4.3 *In vivo* MTP exposure

So far, only few studies are available investigating long-term effects of ß-blockers in fish and most toxicity studies were conducted with concentrations exceeding those expected in the aquatic environment. Furthermore, most studies with ß-blockers have focused on propranolol, a non-specific, very lipophilic ß-blocker. Thus, data gained in this study were mostly compared with studies using ß-blockers other than MTP.

4.3.1 General and morphological parameters

For MTP, van den Brandhof and Montforts (2010) found the NOEC at 12.6 mg L^{-1} in a fish embryo toxicity test with zebrafish. Concentrations as high as 50.5 mg L^{-1} MTP impaired hatching and growth. Results of the study presented here show that chronic exposure to environmentally relevant concentrations of MTP had no effect on hatching success and survival in *O. niloticus*. This is in agreement with findings of Lorenzi et al. (2012). Exposure to 0.1-10 µg L^{-1} propranolol for 21 days did not affect hatchability and time to hatch in fathead minnows. In contrast, growth impairments already occurred at low MTP concentrations in this study. Fish wet weight was reduced dose-dependently after 30 and 80 dph exposure from $4x10^{-10}$ M to $4x10^{-7}$ M MTP. Length was less clear affected by MTP exposure but also dose-dependently reduced after 30 dph. Surprisingly, these observations were not confirmed by gene expression analyses of pituitary GH and hepatic IGF-I. These two main biomarkers of somatic growth did not show any changes in expression patterns due to MTP exposure. Additionally, the CI was only statistically significant reduced after 80 dph comparing $4x10^{-10}$ M (highest CI) and $4x10^{-7}$ M MTP (lowest CI). Propranolol caused a

reduction in fish growth after 14 days exposure to 500 µg L^{-1} in medaka and the number of viable eggs that hatched were decreased already at 0.5 µg L^{-1} (Huggett et al. 2002). Owen et al. (2009) exposed juvenile rainbow trout to sub-lethal levels of propranolol and found a statistically significant reduction of growth during a ten days exposure treatment to 10 mg L^{-1} but also a possible adaptation mechanism as growth recovered with time. In mussels, ten days of propranolol exposure (147 µg L^{-1}) significantly inhibited the feeding rate (Solé et al. 2010). Cardiovascular dysfunction is one possible consequence of exposure to ß-blockers leading to impaired fitness expressed in reduced growth and fecundity (Owen et al. 2007). Triebskorn et al. (2007) analyzed cellular effects in rainbow trout after exposure to 1-100 µg L^{-1} MTP for 28 days and the authors stated that the structural changes found could be a response to compensate for a reduced supply with oxygen and nutrients, which could in turn lead to reduced growth. Catecholamines are important for the mobilization of energy reserves to ensure tissue metabolism during (acute) stress by stimulating gluconeogenesis and glycogenolysis in the liver (Fabbri et al. 1995). If the relevant receptors are blocked (e.g. by MTP), the mobilization could be inhibited. On the other hand, Winter et al. (2008) exposed fathead minnows to atenolol (0.1-10 mg L^{-1}), another selective $ß_1$-blocker, and after 28 days no significant effects on viability, hatching or growth were found in larval fish. 21 days exposure of adult fish had almost the same outcome. In a follow up study, Giltrow et al. (2009) exposed fathead minnows to propranolol (0.001-10 mg L^{-1}) and found mortality and health affected during the first days of the exposure experiment. Taken together, conclusions on the fish toxicity of ß-blockers based on the before mentioned endpoints are mixed and remain somehow unclear.

Calculated HSI did slightly increase with increasing MTP concentrations after 80 dph of exposure but differences were not statistically significant. Bartram et al. (2012) exposed rainbow trout to 1 mg L^{-1} propranolol and did not find a difference in HSI between control and exposed animals neither. The authors also found a general decrease in HSI over the duration of the experiment, as found in Nile tilapia here, and hypothesized that a deprivation of food as found by Metón et al. (2003) could be the reason. The authors linked a reduced liver size to various hepatic changes in gilthead seabream (*Sparus aurata*) including alterations of enzymatic pathways and glycogen levels. Still, no effect of the pharmaceutical treatment was observed. The HSI is only a rough measure for the impacts of pollutants leading to liver growth but if the HSI is almost not changed due to pharmaceutical exposure,

it can be seen as an indicator for rather low toxicity. Again, examination of liver histopathology would give further indication on the toxicity potential.

4.3.2 Histopathological alterations

Exposure to MTP did not affect the number of SLs with histopathological alterations in gills of *O. niloticus* after 80 dph. In fact, $4x10^{-9}$ M MTP and higher concentrations even showed slightly less affected gills and only proliferation of mucus and chloride cells increased dose-dependently. Additionally, infiltrated leucocytes were found significantly more often at $4x10^{-7}$ M MTP compared to the control group. Overall, only mild alterations occurred in a notable amount. These findings are in accordance with findings by Triebskorn et al. (2007). Exposure to similar MTP concentrations in rainbow trout for 28 days caused only little reactions in gills and no clear concentration-effect relationships were found. Epithelial lifting, hyperplasia and hypertrophy of mucus and chloride cells were detected. Avella et al. (1999) could show that an *in vitro* exposure of sea bass (*Dicentrarchus labrax*) gill cells to propranolol blocked the secretion of chloride ions, likely affecting ion regulation in the whole fish. Overall, chronic exposure to environmentally relevant concentrations of MTP affects gill integrity of *O. niloticus* only mild and based on this endpoint, the risk seems to be relatively low.

4.3.3 Gene expression of pituitary gonadotropins and hepatic vitellogenin

Gene expression patterns of the gonadotropins LH and FSH were quite similar after MTP exposure. $4x10^{-10}$ M MTP caused the highest relative expression value und there was a non-significant decrease with increasing concentrations of MTP compared to the control afterwards. Sex steroid levels in Japanese medaka were altered after 14 days of exposure to propranolol (1-500 $\mu g\ L^{-1}$). The level of testosterone was significantly reduced in male fish, whereas E2 levels were significantly increased in both sexes (Huggett et al. 2002). The authors hypothesized that an increased aromatase activity due to the pharmaceutical exposure could be the reason (not tested though). Furthermore, the findings indicate a modified sex steroid regulation. A reduced LH mRNA as found in *O. niloticus* here would also likely lead to a reduced testosterone production via the HPG axis (Milla et al. 2009), confirming the results of Huggett et al. (2002). Further direct measurements of sex steroid levels in blood plasma and aromatase activity on the transcriptional level would help to find more mechanistic explanations for the results gained.

VTG gene expression data complemented the results. Low concentrations of MTP caused only a very little induction of VTG mRNA, only $4x10^{-7}$ M MTP led to a statistically significant increase. Therefore, MTP seems to have the potential to alter endocrine processes but only at concentrations at least 100-fold of the environmentally relevant one in *O. niloticus*. As reviewed by Massarsky et al. (2011), exposure to ß-blockers (mainly propranolol and MTP) affected reproduction, growth, heart rate and hepatic glycogen stores in some fish species. These findings indicate the potential of ß-blockers to acts as EDCs, further strengthened by findings of altered sex hormone levels and delayed embryonic development (Owen et al. 2007). Nonetheless, no data on VTG induction in fish due to MTP exposure is available and direct measures of the endocrine disrupting mode of ß-blockers are rare. Taken together, exposure to environmentally relevant concentrations of MTP has rather mild effects on endpoints discussed in this section.

4.3.4 Detoxification

MTP exposure caused no statistically significant changes in mRNA levels of CPY1A, GST or MDRP in *O. niloticus* at any sampling point. After 80 dph, CYP1A and MDRP showed the tendency to increase with increasing concentrations though. Overall, SDs were quite high in theses data, potentially masking effects. So far, there is good evidence in the literature that CYP1A is involved in the detoxification of ß-blockers in fish (Laville et al. 2004; Bartram et al. 2012) but also in mussels (Contardo-Jara et al. 2010). Even indirect measures indicated this. The structural reactions of the endoplasmic reticulum (severe vesiculation and dilation) found by Triebskorn et al. (2007) in cellular areas adjacent to the hepatic vessels of rainbow trout might indicate an activation of enzymes similar to mammalian cytochromes of the CYP family and therefore an induction of biotransformation mechanisms in the fish liver. Sun et al. (2015) analyzed the transcriptional responses of adult zebrafish exposed to propranolol and MTP (0.03-3 mg L^{-1}) concerning a great variety of genes involved in the detoxification pathways and stress response. Other enzymes were chosen though. In the liver, the authors only found an induction in the expression of hsp70 in male fish, no enzyme involved in detoxification was induced. In marine mussels, propranolol induced phase I metabolism (based on carboxylesterase) statistically significant at 147 µg L^{-1} but GST was reduced compared to the control when exposed to 11 µg L^{-1}. The higher exposure concentrations then led to an increase again (Solé et al. 2010). In the digestive gland of freshwater mussels, no statistically significant effects of seven days of MTP exposure ($2x10^{-9}$ M to $2x10^{-6}$ M) on GST gene expression level were found by Contardo-Jara et al. (2010). Concerning the third

phase of the biotransformation pathway most likely other enzymes than MDRP are involved in the excretion of ß-blockers. Data in fish are almost missing but in mussels P-gps were found to be active after ß-blocker exposure (Fabbri et al. 2009; Contardo-Jara et al. 2010). Combining the results, it seems that environmentally relevant concentrations of MTP (and other ß-blockers) have only little effects on the hepatic detoxification mechanisms in fish.

In gills of *O. niloticus*, no clear pattern was found in gene expression of CYP1A and GST after 80 dph of MTP exposure, too. It seems likely that detoxification of MTP did not occur via the enzymes under investigation. Since histopathological examination did not reveal severe damages of gill tissue other reason such as cytotoxicity or adverse side effects seem unlikely. Bartram et al. (2012) exposed rainbow trout to propranolol *in vivo* (1 mg L^{-1}) and *in vitro* (0.1-400 µg L^{-1}) and analyzed EROD activity (as an indicator for CYP1A activity) in gill and liver samples. *In vivo*, gill EROD activity was statistically significant affected by propranolol and liver samples showed a similar pattern, but non-significant. Environmentally relevant concentrations (0.1 µg L^{-1}) did not statistically significant induce gill or liver EROD activity *in vitro* though, but higher concentrations (200 µg L^{-1}, equivalent to 'normal' human therapeutic level) did. GST gene expression was statistically significant enhanced after four and seven days of exposure to MTP ($2x10^{-7}$ M and $2x10^{-6}$ M) in freshwater mussel *D. polymorpha*, indicating biotransformation processes (Contardo-Jara et al. 2010). In marine mussel (*Mytilus galloprovincialis*), Solé et al. (2010) found a 1.4-fold increase in gill GST expression at a low propranolol (11 µg L^{-1}) concentration, too. Since further data in fish are missing, one can only speculated that detoxification of xenobiotics in gills of vertebrates and invertebrates might be implemented differently.

4.3.5 Oxidative stress

In brain and muscle tissue of *O. niloticus*, a statistically significant increase in TBARS level compared to the control was found due to exposure to $4x10^{-10}$ M and $4x10^{-8}$ M MTP. In gills, no effect was observed. Sample processing was performed at different days and maybe handling issues could give some explanation for these findings (although not found in gills). Furthermore, different responses of organs might be due to different anti-oxidant capacities of the tissues as suggested for the digestive gland and gills in mussels (Solé et al. 2010). Sun et al. (2015) exposed adult zebrafish to propranolol and MTP (0.03-3 mg L^{-1}) and analyzed the transcriptional response of different tissues (brain, liver, gonads) concerning the antioxidant system. Both substances induced the transcriptional response of the antioxidant enzymes

(including CAT and GPx) dose-dependently in both sexes in tissues other than brain. In invertebrates, MTP ($2x10^{-7}$ M and $2x10^{-6}$ M) induced mRNA levels of SOD and CAT statistically significant in the digestive gland but not in the gills of freshwater mussels (Contardo-Jara et al. 2010). In contrast, propranolol exposure (11 µg L^{-1} and 147 µg L^{-1}) resulted in elevated LPO damage in the gills of the marine mussel *M. galloprovincialis* but no evidence for oxidative stress in the digestive gland was found based on CAT and LPO analyses (Solé et al. 2010). To gain more certainty about the effects of MTP exposure on the oxidative stress status in *O. niloticus*, gene expression analyses of certain enzymes such as CAT and SOD would be useful. These could then be compared to available data in other taxa.

4.4 *In vitro* versus *in vivo* preparation

The studies presented here enable a direct comparison of the impacts of pharmaceutical exposure on the detoxification pathway in fish between *in vitro* and *in vivo* preparations. Due to DCF exposure, *in vitro* and *in vivo* gene expression analyses of three key enzymes (CYP1A, GST and MDRP) of the detoxification pathway showed similar patterns, although some differences occurred. The sensitivity of both approaches seems to be comparable, although the increase of CYP1A expression was not statistically significant *in vivo* but *in vitro*. For GST gene expression, a higher increase *in vitro* (up to 4.3-fold) was found while for MDRP the highest increase was found *in vivo* (almost 5-fold). The most prominent difference was found at the highest DCF concentrations ($4x10^{-7}$ M). *In vitro*, all three genes possessed their highest expression level at this concentration while *in vivo*, the expression decreased compared to the second highest concentration. Hypothetically, additional adverse side effects, cell damage or general impairment effects or even adaptation mechanisms could be an explanation. At least, these results indicate that some differences remain when using an *in vitro* or *in vivo* preparation for exposure studies.

In contrast, comparing the gene expression patterns of the *in vitro* and *in vivo* experiments after MTP exposure, less similarities appear. While *in vitro*, CYP1A and GST were statistically significant induced due to MTP exposure, no such significance was found *in vivo*. Nonetheless, $4x10^{-7}$ M MTP caused a statistically significant increase of CYP1A mRNA *in vitro* and *in vivo* the same concentration caused the highest increase of about 3.6-fold compared to the control group as well. Due to high SDs, the increase was non-significant though. *In vitro* GST gene expression was almost twice as high as *in vivo* but MDRP expression levels were comparable between both approaches. Overall, induction of these

detoxification enzymes was rather low and it seems likely that MTP is mainly metabolized through another pathway than investigated.

Concerning the metabolic capability of *O. niloticus* one can derive from the results presented that an *in vitro* approach is as informative as an *in vivo* preparation and in terms of animal welfare the first mentioned is to favor. This is in accordance with findings of Bartram et al. (2012). The authors exposed trout to propranolol and analyzed EROD activity *in vitro* and *in vivo* in gill and liver tissue. In the gills, the *in vitro* EROD induction was generally higher than *in vivo* at similar concentrations, whereas in liver tissue, the induction rate was similar, but only over the initial 24 h of exposure. When comparing rates directly, the authors did not find differences in the EROD activity of the control group between the two approaches. They concluded that *in vitro* preparations could be used as *in vivo* surrogates to study the metabolic capability of fish after exposure to xenobiotics, including pharmaceuticals.

Regarding VTG gene expression, almost the same pattern and level of sensitivity after MTP exposure *in vitro* and *in vivo* were found but due to DCF exposure, VTG gene expression occurred differently. There was a statistically significant increase due to $4x10^{-9}$ M DCF in both preparations. While the increase found *in vitro* was almost dose-dependently, ranging from 1.3-fold to 3-fold, VTG gene expression increased *in vivo* in a NMDR relationship, with a distinct peak at $4x10^{-9}$ M DCF, leading to an inverted U-shape expression pattern. Overall, the increase was 1.5 to 3.8-fold and therefore comparable with the *in vitro* results. Concerning VTG gene expression and therefore the estrogenic mode of action of DCF and MTP, differences between the *in vitro* and *in vivo* preparations occurred. These results illustrate that *in vivo* experiments are necessary to demonstrate the actual relevance of an exposure. Only *in vivo*, all biological effects on the physiology, including detoxification, are taken into account, and therefore other physiological responses of the whole organisms are found.

5 Conclusions and perspectives

Changing (aquatic) environments, naturally or mediated by human activities, challenge the organisms living therein and force them to adapt to altered conditions continuously. The physiological responses, meaning the automatic, instinctive reactions to a stimulus, help the organisms to adapt and survive. Furthermore, they can be examined and be used as indicators for the risks possessed by certain pollutants. Overall, chronic exposure experiments lead to a more realistic risk assessment than acute toxicity tests. The latter often indicate no or very low risk due to very high no observed effect concentrations (NOEC) values that exceed pharmaceutical concentrations found in the aquatic environment. The *in vitro* and *in vivo* studies presented here illustrate valid reasons for the need of chronic, low-dose testing in ecotoxicological research. First of all, some adverse impacts of the pharmaceuticals tested were already found at environmentally relevant concentrations (around $4x10^{-9}$ M). Although hatching and survival, two comprehensive indicators for harmful conditions, were not affected, it was shown that various endpoints were affected that could be of relevance for the population´s viability long-term. Secondly, larvae and juvenile/adult fish were found to possess different sensitivities to certain endpoints and only chronic studies can cover different developmental stages. Finally, non-monotonic dose response relationships can occur (as found for VTG after DCF exposure *in vivo*) and therefore, safe doses cannot always be determined from high doses (Vandenberg et al. 2012).

Concerning the DCF exposure, the results presented here revealed that

(1) ***in vitro*** already environmentally relevant DCF concentrations induced the detoxification and gene expression of CYP1A, GST and MDRP almost dose-dependently.
(2) VTG up-regulation ***in vitro*** demonstrated the estrogenic potential of DCF.
(3) ***in vivo*** no negative effects on hatching success and survival were found. Growth was mildly reduced after 80 dph of exposure, reflected by a non-significant reduction in IGF-I gene expression.
(4) DCF exposure ***in vivo*** caused mild to moderate changes in gill histology and occurring alterations might increase the blood-water diffusion distance and reduce uptake of the xenobiotic.

(5) DCF exposure ***in vivo*** altered gonadotropin and VTG gene expression indicating its estrogenic endocrine activity (Figure 44)

(6) DCF detoxification ***in vivo*** was at least partly catalyzed by a CYP1A-GST-MDRP enzyme route in liver tissue. In gills, there was evidence that the highest DCF concentrations caused cellular damage reducing the detoxification capability and/or other enzymes were more active.

(7) TBARS levels ***in vivo*** were mildly elevated in brain tissue but not in gills and muscles, and therefore the oxidative stress response seems tissue-specific and the risk relatively low.

Concerning MTP exposure, the results presented here revealed that

(1) ***in vitro*** detoxification was only moderately induced due to MTP exposure. Only CYP1A and GST induction was statistically significant.

(2) a mild increase in VTG mRNA ***in vitro*** demonstrated a potential estrogenic mode of action of MTP.

(3) ***in vivo*** hatching success and survival were not affected by MTP exposure but growth was reduced dose-dependently after 30 and 80 dph, respectively.

(4) MTP exposure ***in vivo*** caused mild histopathological alterations, mainly proliferation of mucous/chloride cells were found.

(5) ***in vivo*** the lowest MTP concentration increased LH and FSH mRNA but higher concentrations reduced gene expression. VTG gene expression was induced at the highest MTP concentration and taken together, these results indicate that MTP can alter endocrine regulated processes at certain, relatively high, concentrations (Figure 44).

(6) ***in vivo*** enzymatic activities of CYP1A, GST and MDRP were low and it seems likely that other enzymes are involved in the detoxification of MTP in O. *niloticus* liver and gill tissue.

(7) TBARS levels ***in vivo*** were elevated in brain and muscle tissue indicating that MTP has the potential to cause tissue-specific oxidative stress.

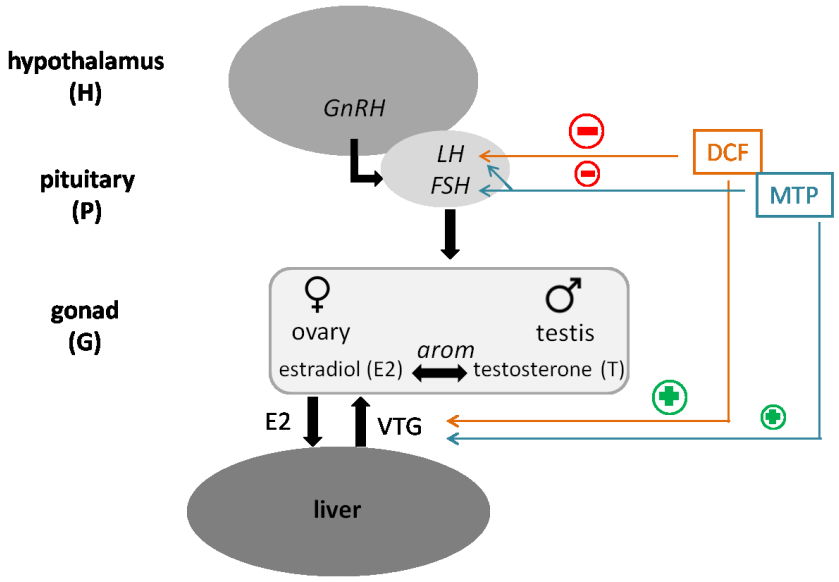


Figure 44. Summary of the effects of diclofenac (DCF) and metoprolol (MTP) on the hypothalamus-pituitary-gonad (HPG) axis of *Oreochromis niloticus*. DCF directly affects synthesis of the pituitary gonadotropin luteinizing hormone (LH) while MTP also affects the follicle stimulating hormone (FSH) but overall less distinct. Both substances induced hepatic vitellogenin (VTG) synthesis but MTP only at higher concentrations compared to DCF. ⊖: inhibition, ⊕: induction, GnRH: gonadotropin releasing hormone, arom: aromatase.

The recent results show that fish possess different targets with different levels of sensitivity concerning exposure to pharmaceuticals such as DCF or MTP. As promoted elsewhere, comprehensive studies should link different relevant endpoints since effective concentrations differ. Furthermore, in future studies the gender of fish should be considered when assessing effects of pharmaceuticals. Not only tissue-specific responses occur, as found in the studies presented, but also gender-specificity might appear. In humans, variations in the metabolism of pharmaceuticals were associated with sex and ethnicity (Sowinski et al. 1996) and different authors found gender-specific responses in fish too (Giltrow et al. 2009; Sun et al. 2015).

Population relevant endpoints as well as gene expression analyses of gonadotropins and detoxification enzymes were the initial focus of this work. Evaluating the endocrine disruption potential of the substances based on VTG gene expression was added. Since at least DCF very likely acts as an EDC, possessing an estrogenic mode of action, future research should focus on sex differentiation, gonadal steroidogenesis, reproductive success and behavioral impacts, e.g. on mating behavior. Additionally, reproductive endpoints such as egg production are especially interesting when studying the effects of ß-blockers, since the reproductive toxicity associated might be highly modified by the physicochemical properties of these compounds, ranging from being very lipophilic to hydrophilic (Overturf et al. 2015).

Furthermore, for ß-blockers, behavior effects should be studied. Owen et al. (2007) already stated that ß-blockers might decrease neural activity in the brain causing abnormal activity rhythms or sleeping and breeding cycles. Furthermore, since pharmaceuticals can act in fish in a similar mode than in mammals, potential side effects such as kidney failure and intestinal pathology should be considered in the future.

Another very interesting approach that should gain more attention is to study the effects of substance mixtures and pharmaceutical exposure combined with other stressors that likely occur in nature. The adrenergic system in fish is particularly important during stress (Wendelaar-Bonga 1997) and certain ß-blockers interfere with the normal physiological functioning. Therefore, the ability of fish to deal with a stressor in the presence of or after a long-term exposure to pharmaceuticals, especially ß-blockers, would be insightful. It is very likely that the stress response would be altered. Certain processes such as the ability to mobilize energy resources as well as the ability to take up and deliver oxygen to the tissues and adjustments of the heart rate and vascular resistance could change with ß-blockade because the involved tissues (or cells) possess ß-ARs. Hence, the survival of the fish could be threatened given the inability to generate the "fight-or-flight" response (Massarsky et al. 2011).

Last but not least, the reversibility of the physiological responses due to pharmaceutical exposure should be examined as done e.g. by Baumann et al. (2014). If the effects would be reversed after a recovery time this would count for an additional incentive to find solutions to eliminate xenobiotics from surface waters especially threatened or were adverse impacts on the aquatic organisms were already detected.

Derived from the results presented, the greatest threat of DCF seems to be its potential to act as an estrogenic endocrine disruptor. For MTP the risk for fish health seems to be low. Mixture effects or effects in combination with other stressors occurring in the aquatic environment most likely lead to a different outcome though, since β-blockers compete for the receptor site of the endogenous catecholamines.

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7 Annex

7.1 DCF measured with LC- QTOF

UPLC parameters:
column: Agilent Eclipse Plus C18 2.1 x 50 mm ; 1.8 µm pore size

solvents:
A: H_2O + 0.1 % formic acid
B: acetonitrile + 0.1 % formic acid
gradient: flow rate 400 µl min^{-1}

Time (min)	A %	B %
0	80	20
10	20	80
11	80	20
12	80	20

injection volumen 1 µL
oven temperature: 30 °C
retention time: 1.5 min

QTOF parameters:
general:

- MRM mode: positive
- ion Source Dual AJS ESI

QTOF MS mode mass range 100-1500 m/z

mass DCF :
DCF M (H+) = 296,0240

hardware :
UPLC : Agilent 1290 Infinity
QTOF : Agilent 6550 iFunnel QTOF LC/MS

software : Agilent Mass Hunter workstation Data Acquisition
Agilent Mass Hunter Qualitative Analysis B.06.00
Agilent Mass Hunter Quantitative Analysis B.06.00

Limit of detection and limit of quantitation were 1 ng mL^{-1} and 25 ng mL^{-1}, respectively. Calibration was linear (R2=) between 25 and 250 ng mL^{-1}.

7.2 MTP measured with LC- QTOF

UPLC- parameters:
column: Agilent Eclipse Plus C18 2.1x50 mm ; 1.8 µm pore size

solvents:
A: H_2O + 0.1 % formic acid
B: acetonitril + 0.1 % formic acid
gradient: flow rate 400 µl min^{-1}

Time (min)	A %	B %
0	95	5
2	20	80
3	95	5
5	95	5

injection volumen 1 µL
column temperature: 20 °C
retention time: 1.5 min

QTOF Parameters:
general:

- positive
- Ion Source Dual AJS ESI

QTOF MS Mode Mass Range 100-1500 m/z

mass MTP :
MTP M (H+) = 268,1908

hardware :
UPLC : Agilent 1290 Infinity
QTOF : Agilent 6550 iFunnel QTOF LC/MS

software : Agilent Mass Hunter workstation Data Acquisition
Agilent Mass Hunter Qualitative Analysis B.06.00
Agilent Mass Hunter Quantitative Analysis B.06.00

Limit of detection and limit of quantitation were 0.2 ng $mL^{-1}$and 1 ng mL^{-1}, respectively. Calibration was linear (R2=) between 1 and 100 ng mL^{-1}.